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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No	SALK1520-2
Total Pages	
First Named Inventor or Application Identifier	Evans et al.
Express Mail Label No.	EM299799893US

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

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1 [X] Fee Transmittal Form
(Submit an original, and a duplicate for fee processing)

2 [X] Specification [Total Pages 90]
(preferred arrangement set forth below)
- Descriptive title of the Invention
- Cross References to Related Applications
- Statement Regarding Fed sponsored R & D
- Reference to Microfiche Appendix
- Background of the Invention
- Brief Summary of the Invention
- Brief Description of the Drawings *(if filed)*
- Detailed Description
- Claim(s)
- Abstract of the Disclosure

3 [X] Drawing(s) *(35 USC 113)* [Total Sheets 6]

4 Oath or Declaration [Total Pages]
a. [ ] Newly executed (original or copy)
b. [ ] Copy from a prior application (37 CFR 1.63(d))
(for continuation/divisional with Box 17 completed)
[Note Box 5 below]
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Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b)

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The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

6 [ ] Microfiche Computer Program *(Appendix)*

7 Nucleotide and/or Amino Acid Sequence Submission
(if applicable, all necessary)
a [ ] Computer Readable Copy
b [ ] Paper Copy (identical to computer copy)
c. [ ] Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

8 [ ] Assignment Papers (cover sheet & document(s))
9 [ ] 37 CFR 3.73(b) Statement *(when there is an assignee)* [ ] Power of Attorney
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11 [ ] Information Disclosure Statement (IDS)/PTO-1449 [ ] Copies of IDS Citations
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A P P L I C A T I O N

for

UNITED STATES LETTERS PATENT

on

Method for Modulating Expression of Exogenous Genes in Mammalian Systems, and Products Related Thereto

by

Ronald M. Evans
David No
Enrique Saez

Sheets of Drawings: Six
Docket No.: SALK1520-2
Salk Ref. No.: S98001

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Methods for Modulating Expression of Exogenous Genes in Mammalian Systems, and Products Related Thereto

RELATED APPLICATIONS

This application is a continuation-in-part of United States Serial No. 08/974,530, filed November 19, 1997, now pending, which is, in turn, a continuation-in-part of United States Serial No. 08/628,830, filed April 5, 1996, now pending, the entire contents of both of which are hereby incorporated by reference herein.

FIELD OF THE INVENTION

The present invention relates to methods in the field of recombinant DNA technology, and products related thereto. More particularly, the invention relates to methods and products for modulating the expression of exogenous genes in mammalian systems.

BACKGROUND OF THE INVENTION

The steroid/thyroid hormone receptors comprise a superfamily of ligand-dependent transcription factors that play a crucial role in mediating changes in cell fate and function (Evans, R.M., *Science* **240**:889-895 (1988)). The receptors transduce extracellular hormonal signals to target genes that contain specific enhancer sequences referred to as hormone response elements (HREs) Evans, (1988); Green and Chambon, *Trends Genet.* **4**:309-314 (1988); Yamamoto, K.R., *Annu. Rev. Genet.* **19**:209-252 (1985)). Each receptor recognizes its own HRE, assuring that a distinct response is triggered by each hormonal signal. Together the collection of related transcription factors and their cognate response elements provides a unique opportunity to control gene expression.

The DNA binding domain of each member of the steroid/thyroid hormone superfamily of receptors has 66-68 amino acids. Twenty of these, including nine cysteines, are conserved throughout the family. The modular structure of members of this receptor superfamily allows the exchange of homologous domains between receptors to create functional chimeras. This strategy was used to demonstrate that the DNA binding domain is solely responsible for the specific recognition of the HRE *in vivo* (Green and Chambon, *Nature* **325**:75-78 (1987); Giguère et al., *Nature* **330**:624-629 (1987); Petkovich et al., *Nature* **330**:444-450 (1987); Kumar et al., *Cell* **51**:941-951 (1987); Umesono et al., *Nature* **336**:262-265 (1988); Thompson and Evans, *Proc. Natl. Acad. Sci. U.S.A.* **86**:3494-3498 (1989) and *in vitro* (Kumar and Chambon, *Cell* **55**:145-156 (1988)). By analogy with the proposed structure for Xenopus transcription factor IIIA (Miller et al., *EMBO J.* **4**:1609-1614 (1985)), the invariant cysteines are thought to form two "zinc fingers" that mediate the DNA binding function (Hollenberg and Evans, *Cell* **55**:899-906 (1988)). Involvement of these cysteines in Zn(II) coordination is supported by extended X-ray absorption fine structure (Freedman et al., *Nature* **334**:543-546 (1988)), and DNA binding by point mutagenesis experiments (Hollenberg and Evans, (1988)); Severne et al., *EMBO J.* **7**:2503-2508 (1988)).

The HREs are in fact structurally related but functionally distinct. The glucocorticoid receptor response element (GRE), estrogen receptor response element (ERE), and thyroid hormone receptor response element (TRE) have been characterized in detail. These particular response elements have been found to have a palindromic pair of hexameric "half-sites" (Evans, (1988); Green and Chambon, (1988)). With optimized pseudo- or consensus response elements, only two nucleotides per half-site differ between GRE and ERE (Klock et al., *Nature* **329**:734-

736 (1987)). On the other hand, EREs and TREs have identical half-sites but the number of nucleotide spacers between the two half sites is different (Glass et al., *Cell* **54**:313-323 (1988)).

In contrast to response elements having the palindromic sequence motif, the following hormone receptors typically recognize response elements having two half-sites in a direct-repeat (DR) sequence motif: RXR, RAR, COUP-TF, PPAR, and the like (see, e.g., Mangelsdorf et al., The Retinoids: Biology, Chemistry, and Medicine, 2nd Edition, Raven Press, Ltd., New York, 1994, Chapter 8). Thus at least three distinct means are used to achieve HRE diversity: 1) binding site specificity for a particular half-site; 2) nucleotide spacing between the two half-sites; and 3) the orientation of the half-sites to one another.

In insect systems, a pulse of the steroid hormone ecdysone triggers metamorphosis in *Drosophila melanogaster* showing genomic effects, such as chromosomal puffing, within minutes of hormone addition. Mediating this response in insects is the functional ecdysone receptor, a heterodimer of the ecdysone receptor (EcR) and the product of the *ultraspiracle* gene (USP) (Yao et al. (1993) *Nature* **366,** 476-479; and Yao et al. (1992) *Cell* **71,** 63-72). Responsiveness to an insect ecdysteroid can be recreated in cultured mammalian cells by co-transfection of EcR, USP, an ecdysone responsive reporter, and treatment with ecdysone or the synthetic analog muristerone A.

In the field of genetic engineering, precise control of gene expression is an invaluable tool in studying, manipulating and controlling development and other physiological processes. For example applications for regulated gene expression in mammalian systems include inducible gene targeting, overexpression of toxic and

teratogenic genes, anti-sense RNA expression, and gene therapy (Jaenisch, R. (1988) *Science* **240,** 1468-1474). For cultured cells, glucocorticoids and other steroids have been used to induce the expression of a desired gene.

As another means for controlling gene expression in a mammalian system, an inducible tetracycline regulated system has been devised and utilized in transgenic mice, whereby gene activity is induced in the absence of the antibiotic and repressed in its presence (see, e.g, Gossen et al. (1992) *Proc. Natl. Acad. Sci.* **89,** 5547-5551; Gossen et al.(1993) *TIBS* **18,** 471-475; Furth et al. (1994) *Proc. Natl. Acad. Sci.* **91,** 9302-9306; and Shockett et al. (1995) *Proc. Natl. Acad. Sci.* **92,** 6522-6526). However, disadvantages of this system include the continuous treatment of tetracycline to repress expression and the slow clearance of antibiotic from bone which interferes with quick and precise induction. While this system has been improved by the recent identification of a mutant tetracycline repressor which acts conversely as an inducible activator, the pharmacokinetics of tetracycline may hinder its use during development when a precise and efficient "on-off" switch is essential (Gossen et al. (1995) *Science* **268,** 1766-1769).

Accordingly, there is a need in the art for improved methods to precisely modulate the expression of exogenous genes in mammalian subjects.

BRIEF DESCRIPTION OF THE INVENTION

In accordance with the present invention, there are provided various methods for modulating the expression of an exogenous gene in a mammalian subject. The invention method is useful in a wide variety of applications where inducible *in vivo* expression of an exogenous gene is desired, such as *in vivo* therapeutic methods for delivering

recombinant proteins into a variety of cells within a patient.

Unlike prior art tetracycline based strategies, transferring ecdysone responsiveness to mammalian cells takes advantage of a naturally evolved steroid inducible system. Advantages of ecdysteroid use include the lipophilic nature of the compounds (which provides efficient penetrance thereof into all tissues, including the brain), short half-lives (which allow for precise and potent inductions), and favorable pharmacokinetics that prevent storage and expedite clearance.

In accordance with another embodiment of the present invention, there are provided modified ecdysone receptors, which can be in the form of homodimeric species or heterodimeric species comprising at least one silent partner of the steroid/thyroid hormone superfamily of receptors, along with an invention modified ecdysone receptor. Invention modified ecdysone receptors are useful, for example, in methods for modulating expression of an exogenous gene in a mammalian subject.

In accordance with additional embodiments of the present invention, there are provided nucleic acids encoding invention modified ecdysone receptors, modified ecdysone receptor response elements, gene transfer vectors, recombinant cells, and transgenic animals containing nucleic acid encoding invention modified ecdysone receptor.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A - 1D show the optimization of ecdysone responsiveness using various combinations of USP or RXR with different modified EcRs. In Figure 1A, the numerical values on both sides of the figure are on the same scale, with the GEcR/RXR value repeated for clarity.

Darkened and stripped bars represent reporter activity with no hormone or 1μM muristerone A, respectively.

Figure 1B shows FXR and VpEcR activity on ecdysone response element (EcRE) and a hybrid ecdysone/glucocorticoid response element (E/GRE) responsive reporters. VpEcR, VgEcR, and control transfection without receptors were treated with 1μM muristerone. FXR transfections were treated with 50μM Juvenile Hormone III (Sigma). Darkened and stripped bars represent reporter activity with no hormone or 1μM muristerone A/50μM Juvenile Hormone III, respectively.

Figure 1C shows that E/GRE and GRE are non-overlapping response elements. Darkened and stripped bars represent reporter activity with no hormone or 1μM muristerone A/1μM dexamethasone, respectively.

Figure 1D shows a schematic diagram of modified ecdysone receptors. GEcR is a chimeric receptor containing the N-terminal transactivation domain of GR and the DNA- and ligand-binding domains of EcR. VpEcR is an N-terminal truncation of EcR, wherein the activation domain of Vp16 is fused thereto at the amino terminus thereof. VgEcR is identical to VpEcR except for the following point mutations in the P box of the DNA binding domain: E282G, G283S, and G286V. Vp16-EcR-B1 is a fusion of full length EcR with the activation domain of Vp16, wherein the activation domain of Vp16 is fused thereto at the carboxy terminus thereof. VgEcR-B1 is identical to Vp16-EcR-B1 except for the same point mutations in the P box of the DNA binding domain as described above. In the Figure, DBD=DNA binding domain and LBD=ligand binding domain.

Figure 2 shows a schematic diagram of an invention ecdysone inducible gene expression system. After

expression of RXR and a modified EcR, the two receptors can heterodimerize and transactivate the ecdysone response element-containing promoter in the presence of hormone. The ecdysone response elements are placed upstream of a minimal promoter (i.e., an enhancerless promoter) which can drive the expression of any exogenous cDNA.

Figure 3A shows a dose-dependent activation of N13 cells with muristerone. N13 cells were grown with varying concentrations of muristerone for 36 hours and then assayed for ß-galactosidase activity (open squares) by standard ONPG assay or for luciferase activity (closed circles). Figure 3B shows the time-course of luciferase activity of N13 cells treated with hormone. N13 cells were grown in separate wells in the presence of 1μM muristerone, harvested at varying times, and assayed for luciferase activity as described in Example 3.

Figure 4 shows muristerone activity in mice as described in Example 4.

Figure 5 compares the dose-dependent activation of N13 cells with muristerone (X) and ponasterone A (open circles).

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there are provided methods for modulating the expression of an exogenous gene in a mammalian subject containing:

(i) a DNA construct comprising said exogenous gene under the control of an ecdysone response element; and

(ii) a modified ecdysone receptor which, in the presence of a ligand therefor, and optionally in the further presence of a

receptor capable of acting as a silent partner therefor, binds to said ecdysone response element;

said method comprising administering to said subject an effective amount of a ligand for said modified ecdysone receptor; wherein said ligand is not normally present in the cells of said subject; and wherein said ligand is not toxic to said subject.

Thus, in accordance with the present invention the insect molting hormone, ecdysone (as well as analogs and mimics thereof), is advantageously employed as a regulated inducer of gene expression in mammalian systems, i.e., background levels of expression are substantially zero in the absence of conditions required for induction. In a presently preferred aspect of the invention, promoters containing a novel modified ecdysone response element are employed in conjunction with an invention modified ecdysone receptor (preferably having an altered DNA binding specificity) to provide an extremely powerful and specific inducible mammalian expression system. The low basal activity of the invention expression system is advantageously suitable for the expression of transcription factors and toxic genes. The excellent dose response and induction rate characteristics of the invention inducible expression system allow for precise control of both the degree and duration of induction of a desired gene.

Since the invention method provides for regulated gene expression by an exogenous non-mammalian inducer, it can be advantageously employed in a variety of *in vivo* and *in vitro* mammalian expression systems. For example, inducible expression of cre recombinase in transgenic mammals, in accordance with invention methods, would enable those of skill in the art to accomplish temporally specific inducible gene targeting of the adult or the developing embryo (O'Gorman et al. (1991) *Science* **251,** 1351-1355).

As employed herein, the terms "modulate" and "modulating" refer to the ability of a given ligand/receptor complex to effect transactivation of transcription of an exogenous gene, relative to such ability of said receptor in the absence of ligand. The actual effect of complex formation on the transactivation activity of a receptor will vary depending on the specific receptor species which are part of the ligand/receptor complex, and on the response element with which the ligand/receptor complex interacts.

As used herein, when referring to genes, the phrase "exogenous to said mammalian subject" or simply "exogenous" refers to any gene wherein the gene product is not naturally expressed in the particular cell where expression is desired. For example, exogenous genes can be either natural or synthetic wild type genes and therapeutic genes, which are introduced into the subject in the form of DNA or RNA. The gene of interest can be introduced into target cells (for *in vitro* applications), or the gene of interest can be introduced directly into a subject, or indirectly introduced by the transfer of transformed cells into a subject.

"Wild type" genes are those that are native to cells of a particular type. Such genes may be undesirably overexpressed, or may not be expressed in biologically significant levels. Thus, for example, while a synthetic or natural gene coding for human insulin would be exogenous genetic material to a yeast cell (since yeast cells do not naturally contain insulin genes), a human insulin gene inserted into a human skin fibroblast cell would be a wild type gene with respect to that cell since human skin fibroblasts contain genetic material encoding human insulin, although human skin fibroblasts do not express human insulin in biologically significant levels.

Wild type genes contemplated for use in the practice of the present invention include genes which encode a gene product:

the substantial absence of which leads to the occurrence of a non-normal state in said subject; or

a substantial excess of which leads to the occurrence of a non-normal state in said subject;

and the like.

As employed herein, the phrase "therapeutic gene" refers to a gene which imparts a beneficial function to the host cell in which such gene is expressed. Therapeutic genes are those that are not naturally found in host cells. For example, a synthetic or natural gene coding for wild type human insulin would be therapeutic when inserted into a skin fibroblast cell so as to be expressed in a human host, where the human host is not otherwise capable of expressing functionally active human insulin in biologically significant levels. In accordance with the methods described herein, therapeutic genes are expressed at a level that provides a therapeutically effective amount of the corresponding therapeutic protein.

Therapeutic genes contemplated for use in the practice of the present invention include genes which encode a gene product:

which is toxic to the cells in which it is expressed; or

which imparts a beneficial property to the host subject (e.g., disease resistance, etc);

and the like.

Numerous genomic and cDNA nucleic acid sequences coding for a variety of proteins are well known in the art. Exogenous genetic material useful in the practice of the

present invention include genes that encode biologically active proteins of interest, such as, e.g., secretory proteins that can be released from said cell; enzymes that can metabolize a substrate from a toxic substance to a non-toxic substance, or from an inactive substance to a useful substance; regulatory proteins; cell surface receptors; and the like. Useful genes include genes that encode blood clotting factors such as human factors VIII and IX; genes that encode hormones such as insulin, parathyroid hormone, luteinizing hormone releasing factor (LHRH), alpha and beta seminal inhibins, and human growth hormone; genes that encode proteins such as enzymes, the absence of which leads to the occurrence of an abnormal state; genes encoding cytokines or lymphokines such as interferons, granulocytic macrophage colony stimulating factor (GM-CSF), colony stimulating factor-1 (CSF-1), tumor necrosis factor (TNF), and erythropoietin (EPO); genes encoding inhibitor substances such as $alpha_1$-antitrypsin; genes encoding substances that function as drugs, e.g., genes encoding the diphtheria and cholera toxins; and the like.

Typically, nucleic acid sequence information for a desired protein can be located in one of many public access databases, e.g., GENBANK, EMBL, Swiss-Prot, and PIR, or in many biology related journal publications. Thus, those of skill in the art have access to nucleic acid sequence information for virtually all known genes. Those of skill in the art can either obtain the corresponding nucleic acid molecule directly from a public depository or the institution that published the sequence. Optionally, once the nucleic acid sequence encoding a desired protein has been ascertained, the skilled artisan can employ routine methods, e.g., polymerase chain reaction (PCR) amplification, to isolate the desired nucleic acid molecule from the appropriate nucleic acid library. Thus, all known nucleic acids encoding proteins of interest are available for use in the methods and products described herein.

As used herein, the terms "mammal" and "mammalian" refer to humans; domesticated animals, e.g., rats, mice, rabbits, canines, felines, and the like; farm animals, e.g., chickens, bovine, porcine and ovine, and the like; and animals of zoological interest, e.g., monkeys and baboons, and the like.

Modified ecdysone receptors contemplated for use in the practice of the present invention comprise:

a ligand binding domain capable of binding an ecdysteroid;

a DNA-binding domain obtained from a DNA-binding protein; and

an activation domain of a transcription factor,

wherein at least one of said DNA-binding domain or said activation domain is not obtained from a native ecdysone receptor,

with the proviso that when said activation domain is derived from a glucocorticoid receptor, said DNA-binding domain is not derived from a glucocorticoid receptor or an *E. coli* LexA protein. In accordance with the present invention, modified ecdysone receptors function in expression systems, preferably mammalian, to transactivate gene expression from transcription regulatory regions having ecdysone response elements. Preferably, in order to minimize induction of undesired gene expression, modified ecdysone receptors of the invention will have substantially no constitutive activity in mammalian cells.

Ligand binding domains capable of binding an ecdysteroid, as contemplated for use in the preparation of invention modified ecdysone receptors are typically derived from the carboxy-terminal portion of native ecdysone receptor and are able to bind ecdysteroids (Koelle et al., *Cell*, **67**:59-77, 1991; and Christopherson et al., *PNAS, USA*,

89:6314-6318, 1992). Ligand binding domains capable of binding an ecdysteroid can be functionally located in either orientation and at various positions within the modified ecdysone receptor of the invention. For example, the ligand binding domain capable of binding an ecdysteroid can be positioned at either the amino or carboxy terminus of the modified receptor, or therebetween. In a preferred embodiment of the present invention, the ligand binding domain capable of binding an ecdysteroid is positioned at the carboxy terminus of the modified receptor (see Figure 1D).

DNA-binding domains contemplated for use in the preparation of invention modified ecdysone receptors are typically obtained from DNA-binding proteins (e.g., transcription factors). The term "DNA-binding domain" is understood in the art to refer to an amino acid sequence that is able to bind to DNA. As used herein, the term "DNA-binding domain" encompasses a minimal peptide sequence of a DNA-binding protein, up to the entire length of a DNA-binding protein, so long as the DNA-binding domain functions to associate with a particular response element.

Such DNA-binding domains are known to function heterologously in combination with other functional protein domains by maintaining the ability to bind the natural DNA recognition sequence (see, e.g., Brent and Ptashne, 1985, Cell, 43:729-736). For example, hormone receptors are known to have interchangeable DNA-binding domains that function in chimeric proteins (see, e.g., U.S. Patent No. 4,981,784; and Evans, R., 1988, Science, 240:889-895). Thus, similar to the ligand binding domain of invention modified ecdysone receptor, the DNA-binding domain can be positioned at either the carboxy terminus or the amino terminus, or the DNA-binding domain can be positioned between the ligand binding domain and the activation domain. In preferred embodiments of the present invention,

the DNA-binding domain is positioned internally between the ligand binding domain and the activation domain.

"DNA-binding protein(s)" contemplated for use herein belong to the well-known class of proteins that are able to directly bind DNA and facilitate initiation or repression of transcription. Exemplary DNA-binding proteins contemplated for use herein include transcription control proteins (e.g., transcription factors and the like; Conaway and Conaway, 1994, "Transcription Mechanisms and Regulation", Raven Press Series on Molecular and Cellular Biology, Vol. 3, Raven Press, Ltd., New York, NY).

Transcription factors contemplated for use herein as a source of such DNA binding domains include, e.g., homeobox proteins, zinc finger proteins, hormone receptors, helix-turn-helix proteins, helix-loop-helix proteins, basic-Zip proteins (bZip), β-ribbon factors, and the like. See, for example, Harrison, S., "A Structural Taxonomy of DNA-binding Domains," Nature, 353:715-719. Homeobox DNA-binding proteins suitable for use herein include, for example, HOX, STF-1 (Leonard et al., 1993, Mol. Endo., 7:1275-1283), Antp, Mat α-2, INV, and the like. See, also, Scott et al. (1989), Biochem. Biophys. Acta, 989:25-48. It has been found that a fragment of 76 amino acids (corresponding to amino acids 140-215 described in Leonard et al., 1993, Mol. Endo., 7:1275-1283) containing the STF-1 homeodomain binds DNA as tightly as wild-type STF-1. Suitable zinc finger DNA-binding proteins for use herein include Zif268, GLI, XFin, and the like. See also, Klug and Rhodes (1987), Trends Biochem. Sci., 12:464; Jacobs and Michaels (1990), New Biol., 2:583; and Jacobs (1992), EMBO J., 11:4507-4517.

Preferably, the DNA-binding domain used herein is obtained from a member of the steroid/thyroid hormone

superfamily of receptors. As used herein, the phrase "member(s) of the steroid/thyroid hormone superfamily of receptors" (also known as "nuclear receptors" or "intracellular receptors") refers to hormone binding proteins that operate as ligand-dependent transcription factors, including identified members of the steroid/thyroid hormone superfamily of receptors for which specific ligands have not yet been identified (referred to hereinafter as "orphan receptors").

Exemplary members of the steroid/thyroid hormone superfamily of receptors (including the various isoforms thereof) include steroid receptors such as glucocorticoid receptor (GR), mineralocorticoid receptor (MR), estrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR), vitamin D_3 receptor (VDR), and the like; plus retinoid receptors, such as the various isoforms of retinoic acid receptor (e.g., RARα, RARβ, or RARγ), the various isoforms of retinoid X receptor (e.g., RXRα, RXRβ, or RXRγ), and the like (see, e.g., U.S. Patent Nos. 4,981,784; 5,171,671; and 5,071,773); thyroid receptors (TR), such as TRα, TRβ, and the like; insect derived receptors such as the ecdysone receptor, and the like; as well as other gene products which, by their structure and properties, are considered to be members of the superfamily, as defined hereinabove, including the various isoforms thereof. Examples of orphan receptors contemplated for use herein as a source of DNA binding domain include HNF4 (see, for example, Sladek et al., in Genes & Development 4: 2353-2365 (1990)), the COUP family of receptors (see, for example, Miyajima et al., in Nucleic Acids Research 16: 11057-11074 (1988), and Wang et al., in Nature 340: 163-166 (1989)), COUP-like receptors and COUP homologs, such as those described by Mlodzik et al., in Cell 60: 211-224 (1990) and Ladias et al., in Science 251: 561-565 (1991), various isoforms of peroxisome

proliferator-activated receptors (PPARs; see, for example, Issemann and Green, supra), the insect derived knirps and knirps-related receptors, and the like.

The DNA-binding domains of all members of the steroid/thyroid hormone superfamily of receptors are related, consisting of 66-68 amino acid residues, and possessing about 20 invariant amino acid residues, including nine cysteines. A member of the superfamily can be characterized as a protein which contains these 20 invariant amino acid residues. The highly conserved amino acids of the DNA-binding domain of members of the superfamily are as follows:

Cys - X - X - Cys - X - X - Asp* - X -
Ala* - X - Gly* - X - Tyr* - X - X -
X - X - Cys - X - X - Cys - Lys* - X -
Phe - Phe - X - Arg* - X - X - X - X -
X - X - X - X - X - (X - X -) Cys - X
-X - X - X - X - (X - X - X -) Cys - X
-X - X - Lys - X - X - Arg - X - X -
Cys - X - X - Cys - Arg* - X - X -
Lys* - Cys - X - X - X - Gly* - Met
(SEQ ID NO:1);

wherein X designates non-conserved amino acids within the DNA-binding domain; an asterisk denotes the amino acid residues which are almost universally conserved, but for which variations have been found in some identified hormone receptors; and the residues enclosed in parenthesis are optional residues (thus, the DNA-binding domain is a minimum of 66 amino acids in length, but can contain several additional residues).

Modification of existing DNA-binding domains to recognize new target recognition sequences is also contemplated herein. For example, in accordance with the

present invention, it has been found that the modification of the "P-box" sequence of DNA-binding domains of members of the steroid/thyroid hormone superfamily of receptors offers unique advantages not present in other chimeric hormone receptors. For example, the modification of a P-box amino acid sequence to preferentially bind to a different hormone response element half-site than the naturally occurring P-box amino acid sequence can reduce undesired background levels of gene expression. Thus, invention receptors and methods provide the advantage of increasing the selectivity of exogenous gene expression in a particular subject.

As used herein, the phrase "P-box amino acid sequence" refers to the proximal element region in a DNA-binding domain of a hormone receptor that typically occurs at the junction of the first zinc finger and the linker region, e.g., at about amino acids 19-23 of the DNA-binding domain (i.e., amino acids 19-23 of SEQ ID NO:1; see, e.g., Umesono et al. (1989), Cell, **57**:1139-1146, Figure 2). Umesono et al. (1989), supra, in Table 1, describe various naturally occurring P-box amino acid sequences for a variety of hormone receptor DNA-binding domains.

In one embodiment of the present invention, the P-box sequence of a hormone receptor DNA-binding domain is modified to have a P-box amino acid sequence that differs from the naturally occurring P-box amino acid sequence. In a preferred embodiment of the present invention, the modified P-box amino acid sequence differs from the naturally occurring P-box amino acid sequence by 3 amino acids.

Preferably, the P-box amino acid sequence is modified so that only the half-site nucleotide sequence recognized by the DNA-binding domain is changed while not altering the spacing between the two half-sites recognized

by the DNA-binding domain. For example, when the DNA-binding domain of the ecdysone receptor is employed in an invention modified ecdysone receptor, the P-box can be modified from the amino acid sequence EGCKG (SEQ ID NO:2; which recognizes the half-site -AGGTCA-) to the amino acid sequence GSCKV (SEQ ID NO:3; which recognizes the half-site seqeunce -AGAACA-). In a presently preferred embodiment, when the DNA-binding domain of invention modified ecdysone receptor is derived from ecdysone receptor, the P-box amino acid sequence is modified to GSCKV (SEQ ID NO:3).

It has also been found that *in vitro* evolution methods can be applied to modify and improve existing DNA-binding domains (see, e.g., Devlin et al., 1990, Science, 249:404-406; and Scott and Smith, 1990, Science, 249:386-390).

Activation domains contemplated for use in the preparation of invention modified ecdysone receptor are typically derived from transcription factors and comprise a contiguous sequence of amino acids that functions to activate gene expression when associated with a suitable DNA-binding domain and a suitable ligand binding domain. As with the ligand and DNA-binding domains employed for the preparation of invention modified ecdysone receptors, the activation domain can be positioned at the carboxy terminus, the amino terminus or between the ligand binding domain and the DNA binding domain. In preferred embodiments of present invention, the activation domain is positioned at the amino terminus or the carboxy terminus of the modified ecdysone receptor.

Suitable activation domains can be obtained from a variety of sources, e.g., from the N-terminal region of a member of the steroid/thyroid hormone superfamily of receptors, from a transcription factor activation domain, such as, for example, VP16 or GAL4 activation domains, and

the like. The presently most preferred activation domain contemplated for use in the practice of the present invention is obtained from the N-terminal region of the VP16 protein.

The presently most preferred modified ecdysone receptors contemplated for use herein are VgEcR (SEQ ID NO:5), VpEcR (SEQ ID NO:7), GEcR (SEQ ID NO:9), Vp16-EcR-B1 or VgEcR-B1, with VgEcR (SEQ ID NO:5) and VgEcR-B1 being especially preferred. The preparation of several of these modified ecdysone receptors is set forth hereinafter in Example 1. See also Figure 1D. Those modified receptors for which explicit methods of preparation is not provided herein can readily be made using the methodology set forth herein in combination with standard methodology well known to those of skill in the art.

Invention modified ecdysone receptor proteins can be produced by expressing nucleic acid constructs encoding the chimeric proteins in suitable host cells as described in Example 1. Recombinant methods of producing desired proteins by introducing an expression construct into appropriate host cells are well-known in the art. Modified ecdysone receptors of the invention can be introduced into a particular subject by direct introduction of the proteins themselves, by introducing DNA construct(s) encoding the receptor into the subject, or into cells obtained from the subject (wherein the cells are transformed and subsequently returned to the subject).

In a preferred embodiment, invention modified ecdysone receptors are expressed under the control of a tissue specific promoter. As readily understood by those of skill in the art, the term "tissue specific" refers to the substantially exclusive initiation of transcription in the tissue from which a particular promoter drives expression of a given gene.

In accordance with one aspect of the present invention, invention modified ecdysone receptors are present in the form of heterodimeric species comprising an invention modified ecdysone receptor and at least one silent partner of the steroid/thyroid hormone superfamily of receptors. Preferably, the silent partner is a mammalian-derived receptor, with RXR being especially preferred.

Silent partners contemplated herein are members of the steroid/thyroid hormone superfamily of receptors which are capable of forming heterodimeric species with the invention modified ecdysone receptor, wherein the silent partner does not directly participate in binding ligand (i.e., only the modified ecdysone receptor co-partner of the heterodimer binds ligand). The silent partner can either be endogenous to the cells of the subject or can be provided to the subject by introducing DNA construct(s) encoding receptor into the subject. A preferred silent partner for use herein is RXR. In a particular embodiment of the invention methods, exogenous RXR is provided to said mammalian subject.

The formation of heterodimeric receptor(s) can modulate the ability of member(s) of the steroid/thyroid hormone superfamily of receptors to trans-activate transcription of genes maintained under expression control in the presence of ligand for said receptor. For example, formation of a heterodimer of the modified ecdysone receptor with another mammalian hormone receptor promotes the ability of the modified ecdysone receptor to induce trans-activation activity in the presence of an ecdysone response element.

In accordance with another aspect of the present invention, invention modified ecdysone receptors are

present in the form of homodimeric species comprising a plurality (i.e., at least two) invention modified ecdysone receptors.

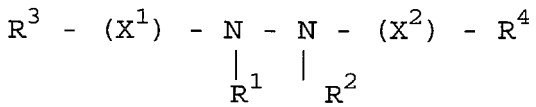
Ligands contemplated for use herein are compounds which, inside a cell, bind to invention modified ecdysone receptors, thereby creating a ligand/receptor complex, which in turn can bind to an appropriate response element. The terms "ecdysone", "ecdysteroid", "ecdysone-analogs", and "ecdysone mimics" as interchangeably used herein, are employed herein in the generic sense (in accordance with common usage in the art), referring to a family of ligands with the appropriate binding and transactivation activity (see, for example, Cherbas et al., in *Biosynthesis, metabolism and mode of action of invertebrate hormones* (ed. J. Hoffmann and M. Porchet), p. 305-322; Springer-Verlag, Berlin). An ecdysone, therefore, is a steroid, steroid-like or non-steroidal compound which acts to modulate gene transcription for a gene maintained under the control of a suitable response element, as described herein.

20-Hydroxy-ecdysone (also known as β-ecdysone) is the major naturally occurring ecdysone. Unsubstituted ecdysone (also known as α-ecdysone) is converted in peripheral tissues to β-ecdysone. Analogs of the naturally occurring ecdysones are also contemplated within the scope of the present invention. Examples of such analogs, commonly referred to as ecdysteroids, include ponasterone A, ponasterone B, ponasterone C, ponasterone D, 26-iodoponasterone A, muristerone A, inokosterone, 26-mesylinokosterone, sidasterone, buterosterone, ajugasterone, makisterone, cyasterone, sengosterone, and the like. Since it has been previously reported that the above-described ecdysones are neither toxic, teratogenic, nor known to affect mammalian physiology, they are ideal

candidates for use as inducers in cultured cells and transgenic mammals according to the invention methods.

Additional compounds contemplated for use herein are mimics of the naturally occurring ecdysones, i.e., synthetic organic compounds which have binding and transactivation activities characteristic of the naturally occurring ecdysones. Examples of such compounds, referred to herein as ecdysone mimics, include 1,2-diacyl hydrazines (e.g., those described in U.S. Patent Nos. 5,424,333 and 5,354,762, the entire contents of each of which are hereby incorporated by reference herein), N'-substituted-N,N'-di-substituted hydrazines (e.g., those described in U.S. Patent No. 5,117,057, the entire contents of which are hereby incorporated by reference herein), dibenzoylalkyl cyanohydrazines (e.g., those described in European Application No. 461,809, the entire contents of which are hereby incorporated by reference herein), N-substituted-N-alkyl-N,N'-diaroyl hydrazines (e.g., those described in U.S. Patent No. 5,225,443, the entire contents of which are hereby incorporated by reference herein), N-substituted-N-acyl-N-alkyl, carbonyl hydrazines (e.g., those described in European Application No. 234,944, the entire contents of which are hereby incorporated by reference herein), N-aroyl-N'-alkyl-N'-aroyl hydrazines (e.g., those described in U.S. Patent No. 4,985,461, the entire contents of which are hereby incorporated by reference herein), and the like.

Compounds of specific interest are those having the formula:



wherein:

R^1 is optionally hydrogen, lower alkyl or substituted lower alkyl, alkenyl or substituted alkenyl, alkynyl or substituted alkynyl, aryl or substituted aryl, heteroaryl or substituted heteroaryl, and the like. R^1 is not present when X^1 is part of a carbon-nitrogen double bond linking R^3 to the hydrazino group.

R^2 is optionally hydrogen, alkyl or substituted alkyl, cyclohexyl or substituted cyclohexyl, and the like. R^2 is not present when X^2 is part of a carbon-nitrogen double bond linking R^4 to the hydrazino group.

R^3 and R^4 are independently part of an appropriately substituted carbon-nitrogen double bond which links R^3 and/or R^4 to the hydrazino linkage, or R^3 and R^4 are independently aryl or substituted aryl, heteroaryl or substituted heteroaryl, provided, however, that when two adjacent positions on the aryl or heteroaryl moieties are substituted with alkoxy, thioalkyl, alkylamino, or dialkylamino groups, these groups may be joined to form a 5- or 6- membered heterocyclic ring system, or R^3 and R^4 are independently heterocyclic or substituted heterocyclic, cycloalkyl or substituted cycloalkyl, and the like.

X^1 and X^2 are independently -C(O)-, -C(S)-, $-C(NR_2)-$, -C(=CN)NH-, -C(O)O-, -C(O)NH-, $-C(O)NHSO_2-$, $-CH_2-$, $-SO_2-$, $-P(O)CH_3-$, and the like, as well as an appropriate substituted carbon-nitrogen double bond which links R^3 and/or R^4 to the hydrazino linkage.

As employed herein, "alkyl" refers to alkyl groups having in the range of 1 up to 8 carbon atoms; "lower alkyl" refers to alkyl groups having in the range of 1 up to 4 carbon atoms; and "substituted alkyl" or "substituted lower alkyl" comprises alkyl (or lower alkyl) groups further bearing one or more substituents selected from halogen, cyano, nitro, hydroxy, alkoxy (-OR),

thioalkyl (-SR), $-NR_2$, -NRC(O)R, -OC(O)R, -C(O)OR, $-C(O)NR_2$, -C(O)R, wherein each R is independently hydrogen or lower alkyl, and the like.

As employed herein, "cycloalkyl" refers to cyclic ring-containing groups containing in the range of about 5 up to 8 carbon atoms, and "substituted cycloalkyl" refers to cycloalkyl groups further bearing one or more substituents as set forth above, as well as lower alkyl.

As employed herein, "heterocyclic" refers to cyclic (i.e., ring-containing) groups containing one or more (up to four) heteroatoms (e.g., N, O, S, or the like) as part of the ring structure, and having in the range of 2 up to 5 nuclear carbon atoms and "substituted heterocyclic" refers to heterocyclic groups further bearing one or more substituents as set forth above, as well as lower alkyl.

As employed herein, "alkenyl" refers to straight or branched chain hydrocarbyl groups having at least one carbon-carbon double bond, and having in the range of about 2 up to 12 carbon atoms, and "substituted alkenyl" refers to alkenyl groups further bearing one or more substituents as set forth above.

As employed herein, "alkynyl" refers to straight or branched chain hydrocarbyl groups having at least one carbon-carbon triple bond, and having in the range of about 2 up to 12 carbon atoms, and "substituted alkynyl" refers to alkynyl groups further bearing one or more substituents as set forth above.

As employed herein, "aryl" refers to aromatic groups having in the range of 6 up to 14 carbon atoms and "substituted aryl" refers to aryl groups further bearing one or more substituents as set forth above, as well as lower alkyl.

As employed herein, "heteroaryl" refers to aromatic groups containing one or more heteroatoms (e.g., N, O, S, or the like) as part of the ring structure, and having in the range of 3 up to 14 carbon atoms and "substituted heteroaryl" refers to heteroaryl groups further bearing one or more substituents as set forth above.

Presently preferred ecdysone mimics contemplated for use herein include compounds wherein R^1 is hydrogen; R^2 is an alkyl group possessing considerable bulk (such as, for example, alkyl groups containing a tertiary carbon center, e.g., $-C(R")_3$, wherein each R" is methyl or greater). Examples of alkyl groups having sufficient bulk for use herein include tert-butyl, sec-butyl, isopropyl, isobutyl, cyclohexyl, cyclopentyl, dicyclopropylmethyl, (cyclohexyl)ethyl, and the like); X^1 and X^2 are both -C(O)-; R^3 is phenyl, substituted phenyl (with hydroxy, alkoxy, halo and/or substituted amino substituents being preferred, with 3,4-disubstitution pattern being especially preferred), heterocyclic (e.g., pyridyl or pyrimidine) or substituted heterocyclic (with halo, alkyl, thioalkyl, hydroxy, alkoxy, and/or amino substituents being preferred); and R^4 is phenyl or substituted phenyl, heteroaryl or substituted heteroaryl or a bulky alkyl or cycloalkyl group.

Especially preferred ecdysone mimics contemplated for use herein include N'-(3,5-dimethylbenzoyl)-N-(4-ethylbenzoyl)-N'-(tert-butyl) hydrazine, N,N'-dibenzoyl-N'-(tert-butyl) hydrazine, N'-(3,5-dimethylbenzoyl)-N-(4-ethylbenzyl)-N'-(tert-butyl) hydrazine, N'-(3,5-dimethylbenzoyl)-N-(2-methyl-3,4-(ethylenedioxy)-benzoyl)-N'-(tert-butyl) hydrazine, 3,5-di-tert-butyl-4-hydroxy-N-isobutyl-benzamide, 8-O-acetylharpagide, and the like.

Ligands contemplated for use in the practice of the present invention are characterized as not normally being present in the cells of the subject, meaning that the ligand is exogenous to the subject. Ecdysteroids, for example, are not naturally present in mammalian systems. Thus, in accordance with the invention method, unless and until an ecdysteroid is administered to the subject, substantially no expression of the desired gene occurs.

An effective amount of ligand contemplated for use in the practice of the present invention is the amount of ligand (i.e., ecdysteroid) required to achieve the desired level of gene expression product. Ligand can be administered in a variety of ways, as are well-known in the art. For example, such ligands can be administered topically, orally, intravenously, intraperitoneally, intravascularly, and the like.

As readily recognized by those of skill in the art, it may be desirable to be able to rapidly induce or rapidly turn off expression by the invention expression system. This can readily be accomplished by administration of a suitable ecdysone antagonist before or after induction of the system (e.g., to prevent undesired activation of the system, to promote rapid induction, to rapidly terminate expression, and the like). Numerous ecdysone antagonists are known in the art, e.g., ajugalactone.

In accordance with a particular embodiment of the present invention, pharmaceutically acceptable formulations, and kits thereof, comprising at least one ecdysteroid, and a pharmaceutically acceptable carrier are contemplated. In accordance with another aspect of the present invention, pharmaceutically acceptable formulations consisting essentially of at least one ecdysteroid and a pharmaceutically acceptable carrier, are contemplated.

Pharmaceutical formulations of the present invention can be used in the form of a solid, a solution, an emulsion, a dispersion, a micelle, a liposome, and the like, wherein the resulting formulation contains one or more of the ecdysteroids of the present invention, as an active ingredient, in admixture with an organic or inorganic carrier or excipient suitable for enteral or parenteral applications.

The active ingredient may be compounded, for example, with the usual non-toxic, pharmaceutically acceptable carriers suitable for oral, topical, nasal, transdermal, intravenous, subcutaneous, intramuscular, intracutaneous, intraperitoneally, intravascular and the like administration. Administration in the form of creams, lotions, tablets, dispersible powders, granules, syrups, elixirs, sterile aqueous or non-aqueous solutions, suspensions or emulsions, and the like, is contemplated. Exemplary pharmaceutically acceptable carriers include carriers for tablets, pellets, capsules, suppositories, solutions, emulsions, suspensions, and any other form suitable for use. Such carriers which can be used include glucose, lactose, gum acacia, gelatin, mannitol, starch paste, magnesium trisilicate, talc, corn starch, keratin, colloidal silica, potato starch, urea, medium chain length triglycerides, dextrans, and other carriers suitable for use in manufacturing preparations, in solid, semisolid, or liquid form. In addition auxiliary, stabilizing, thickening and coloring agents and perfumes may be used. The active compound (i.e., ecdysteroid as described herein) is included in the pharmaceutically acceptable formulation in an amount sufficient to produce the desired effect upon the process or condition of diseases.

Pharmaceutically acceptable formulations containing the active ingredient may be in a form suitable for oral use, for example, as aqueous or oily suspensions,

syrups or elixirs, tablets, troches, lozenges, dispersible powders or granules, emulsions, or hard or soft capsules. For the preparation of oral liquids, suitable carriers include emulsions, solutions, suspensions, syrups, and the like, optionally containing additives such as wetting agents, emulsifying and suspending agents, dispersing agents, sweetening, flavoring, coloring, preserving and perfuming agents, and the like. Formulations intended for oral use may be prepared according to any method known to the art for the manufacture of pharmaceutically acceptable formulations.

Tablets containing the active ingredient in admixture with non-toxic pharmaceutically acceptable excipients may also be manufactured by known methods. The excipients used may be, for example, (1) inert diluents such as calcium carbonate, lactose, calcium phosphate or sodium phosphate; (2) granulating and disintegrating agents such as corn starch, potato starch or alginic acid; (3) binding agents such as gum tragacanth, corn starch, gelatin or acacia, and (4) lubricating agents such as magnesium stearate, stearic acid or talc. The tablets may be uncoated or they may be coated by known techniques to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glyceryl monostearate or glyceryl distearate may be employed. They may also be coated by the techniques described in the U.S. Pat. Nos. 4,256,108; 4,160,452; and 4,265,874, to form osmotic therapeutic tablets for controlled release.

In some cases, formulations for oral use may be in the form of hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin. They may also be in the form of soft gelatin capsules

wherein the active ingredient is mixed with water or an oil medium, for example, peanut oil, liquid paraffin, or olive oil.

The pharmaceutically acceptable formulations may be in the form of a sterile injectable suspension. Suitable carriers include non-toxic parenterally-acceptable sterile aqueous or non-aqueous solutions, suspensions, or emulsions. This suspension may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. They can also be manufactured in the form of sterile water, or some other sterile injectable medium immediately before use. Sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or diglycerides, fatty acids (including oleic acid), naturally occurring vegetable oils like sesame oil, coconut oil, peanut oil, cottonseed oil, etc., or synthetic fatty vehicles like ethyl oleate or the like. They may be sterilized, for example, by filtration through a bacteria-retaining filter, by incorporating sterilizing agents into the formulations, by irradiating the formulations, or by heating the formulations. Sterile injectable suspensions may also contain adjuvants such as preserving, wetting, emulsifying, and dispersing agents. Buffers, preservatives, antioxidants, and the like can be incorporated as required.

Compounds contemplated for use in the practice of the present invention may also be administered in the form of suppositories for rectal administration of the drug. These formulations may be prepared by mixing the drug with a suitable non-irritating excipient, such as cocoa butter, synthetic glyceride esters of polyethylene glycols, which are solid at ordinary temperatures, but liquefy and/or dissolve in the rectal cavity to release the drug.

The pharmaceutically acceptable formulations are administered in a manner compatible with the route of administration, the dosage formulation, and in a therapeutically effective amount. The required dosage will vary with the particular treatment desired, the degree and duration of therapeutic effect desired, the judgment of the practitioner, as well as properties peculiar to each individual. Moreover, suitable dosage ranges for systemic application depend on the route of administration. It is anticipated that dosages between about 10 micrograms and about 1 milligram per kilogram of body weight per day will be used for therapeutic treatment.

An effective amount of the pharmaceutically acceptable formulation contemplated for use in the practice of the present invention is the amount of the pharmaceutically acceptable formulation (e.g., ecdysteroids(s)) required to achieve the desired level of transcription and/or translation of exogenous nucleic acid. A therapeutically effective amount is typically an amount of a ligand or ligand precursor that, when administered in a pharamceutically acceptable formulation, is sufficient to achieve a plasma concentration of the transcribed or expressed nucleic acid product from about 0.1 µg/ml to about 100 µg/ml, preferably from about 1.0 µg/ml to about 50 µg/ml, more preferably at least about 2 µg/ml and usually 5 to 10 µg/ml.

Pharmaceutically acceptable formulations containing suitable ligand(s) are preferably administered intravenously, as by injection of a unit dose, for example. The term "unit dose," when used in reference to a pharmaceutically acceptable formulation of the present invention, refers to a quantity of the pharmaceutical formulation suitable as unitary dosage for the subject, each unit containing a predetermined quantity of active

material calculated to produce the desired therapeutic effect in association with the required diluent, i.e., carrier, or vehicle. It may be particularly advantageous to administer such formulations in depot or long-lasting form as discussed hereinafter.

Suitable regimes for initial administration and booster shots are variable, but are typified by an initial administration followed by repeated doses at one or more intervals by a subsequent injection or other administration. Alternatively, continuous intravenous infusion sufficient to maintain concentrations in the blood in the ranges specified for *in vivo* therapies are contemplated.

Ecdysone response elements contemplated for use in the practice of the present invention (relating to modulation of the expression of exogenous genes in a subject) include native, as well as modified ecdysone response elements. Since invention modified ecdysone receptors can function as either homodimers or as heterodimers (with a silent partner therefor), any response element that is responsive to an invention modified ecdysone receptor, in the form of a homodimer or heterodimer, is contemplated for use in the invention methods described herein. As is readily recognized by those of skill in the art, modified receptors according to the invention (whether in the form of a homodimer or a heterodimer) can bind to either a response element having an inverted repeat motif (i.e., two or more half sites in mirror image orientation with respect to one another), or to a response element having a direct repeat motif.

In a preferred embodiment of the invention, invention modified ecdysone response elements are engineered so as to no longer be capable of binding to a farnesoid hormone receptor (since the mammalian farnesoid

hormone receptor is able to bind to native ecdysone receptor response element). Invention modified ecdysone response elements provide low background expression levels of the exogenous gene and increase the selectivity of the gene expression system when used in mammalian systems.

Ecdysone response elements contemplated for use herein are short cis-acting sequences (i.e., having about 12-20 bp) that are required for activation of transcription in response to a suitable ligand, such as ecdysone or muristerone A, associated with a particular hormone receptor. The association of these response elements with otherwise ecdysone-nonresponsive regulatory sequences causes such regulatory sequences to become ecdysone responsive. Ecdysone response element sequences function in a position- and orientation-independent fashion.

The native ecdysone response element has been previously described, see, e.g., Yao et al., Cell, **71**:63-72, 1992. Modified ecdysone response elements according to present invention comprise two half-sites (in either direct repeat or inverted repeat orientation to one another), separated by a spacer of 0-5 nucleotides. As used herein, the term "half-site" refers to a contiguous 6 nucleotide sequence that is bound by a particular member of the steroid/thyroid hormone superfamily of receptors. Each half-site is typically separated by a spacer of 0 up to about 5 nucleotides. Typically, two half-sites with a corresponding spacer make up a hormone response element. Hormone response elements can be incorporated in multiple copies into various transcription regulatory regions.

Preferred modified ecdysone response elements according to the invention comprise, in any order, a first half-site and a second half-site separated by a spacer of 0-5 nucleotides;

wherein the first and second half-sites are inverted with respect to each other;

wherein said first half-site has the sequence:

-RGBNNM-,

(or complements thereof) wherein

each R is independently selected from A or G;

each B is independently selected from G, C, or T;

each N is independently selected from A, T, C, or G; and

each M is independently selected from A or C;

with the proviso that at least 4 nucleotides of each -RGBNNM- group of nucleotides are identical with the nucleotides at comparable positions of the sequence -AGGTCA-; and

said second half-site is obtained from a glucocorticoid receptor subfamily response element.

The complement to the -RGBNNM- sequence set forth above is:

-YCVNNK-,

wherein

each Y is independently selected from T or C;

each V is independently selected from C, G, or A;

each N is independently selected from A, T, C, or G; and

each K is independently selected from T or G.

Exemplary first half-sites having the -RGBNNM- motif for use in the invention modified ecdysone response element include, for example, half-sites selected from -AGGGCA-, -AGTTCA-, -AGGTAA-, -AGGTCA-, -GGTTCA-, -GGGTTA-, -GGGTGA-, -AGGTGA-, or -GGGTCA-. A particularly preferred first half-site is -AGTGCA-.

Glucocorticoid receptor subfamily response elements contemplated for use in the practice of the

present invention are response elements having half-sites that are typically bound by glucocorticoid, mineralocorticoid, progesterone or androgen receptors. Suitable half-sites from glucocorticoid receptor subfamily response elements can be selected from the following sequence (in either orientation):

-RGNNCA-

(or complements thereof such as -YCNNGT-), wherein R, Y and N are as defined above. Exemplary half-sites having the -RGNNCA- motif for use in the invention modified ecdysone response element include -AGAACA-, -GGAACA-, -AGTTCA-, -AGGTCA-, -GGAACA-, -GGTTCA-, -GGGTCA-, -GGGTCA-, -AGGTGA-, -GGGTCA-, and the like, as well as complements thereof. Particularly preferred half-sites having the -RGNNCA- motif include -AGAACA- and -GGAACA-, with -AGAACA- being especially preferred.

When the above-described modified ecdysone response elements are employed to bind invention heterodimeric receptors, the second half-site is inverted with respect to the first half-site. For example, when describing a single-strand of an invention modified ecdysone response element in the 5'-3' direction, the following general motif can be employed:

RGBNNM-$(N)_x$-TGNNCY (SEQ ID NO:10),

where x is an integer of 0 up to about 5, with x = 1 being especially preferred. As an alternative orientation to the above described response element motif (SEQ ID NO:10), an invention response element can be described in the 5'-3' direction as:

RGNNCA-$(N)_x$-KNNVCY (SEQ ID NO:11),

where x is an integer of 0 up to about 5, with x = 1 being especially preferred.

In preferred embodiments of the present invention, the first half-site is obtained from an ecdysone response element and the second half-site is obtained from a hormone response element selected from a glucocorticoid response element, a mineralocorticoid response element, a progesterone response element or an androgen response element. In a particularly preferred embodiment of the present invention, the first half-site is obtained from an ecdysone response element and the second half-site is obtained from a glucocorticoid response element.

In a particularly preferred embodiment of the invention modified ecdysone response element, the first half-site is AGTGCA and said second half-site is TGTTCT. The presently most preferred modified-ecdysone response element for use in the invention methods is:

AGTGCA-N-TGTTCT (SEQ ID NO:12).

In another aspect of the invention, when modified ecdysone receptors of the invention exist as homodimers, response elements employed preferably have a direct repeat motif (instead of the above-described inverted repeat motif), as follows:

RGBNNM-$(N)_{x'}$-RGBNNM (SEQ ID NO:13),

where R, B, N and M are as previously defined, and x' is an integer of 0 up to about 5, with x' = 3 being especially preferred.

Invention modified ecdysone response elements are characterized as having substantially no constitutive activity, which refers to the substantial absence of background levels of gene expression initiated by invention modified ecdysone response elements when introduced into

mammalian expression systems. Since it has been found that mammalian farnesoid hormone receptors are able to bind to and transactivate gene expression from native ecdysone response elements, in certain embodiments of the present invention (e.g., where it is desired to avoid farnesoid-mediated background expression), modified ecdysone response elements are employed.

Presently preferred invention modified ecdysone response elements are further characterized as having substantially no binding affinity for farnesoid X receptor (FXR), i.e., invention response elements are incapable of binding FXR (which would thereby create undesired background levels of expression). Thus, presently preferred invention modified ecdysone response elements preferably induce basal levels of expression of substantially zero.

Response elements employed in the practice of the present invention are operably linked to a suitable promoter for expression of exogenous gene product(s). As used herein, the term "promoter" refers to a specific nucleotide sequence recognized by RNA polymerase, the enzyme that initiates RNA synthesis. This sequence is the site at which transcription can be specifically initiated under proper conditions. When exogenous genes, operatively linked to a suitable promoter, are introduced *into the* cells of a suitable host, expression of the exogenous genes is controlled by the presence of ecdysteroid compounds, which are not normally present in the host cells.

In accordance with another embodiment of the present invention, there are provided methods of inducing the expression of an exogenous gene in a mammalian subject containing:

(i) a DNA construct comprising an exogenous gene under the control of an ecdysone response element,

(ii) DNA encoding a modified ecdysone receptor under the control of an inducible promoter; wherein said modified ecdysone receptor, in the presence of a ligand therefor, and optionally in the further presence of a receptor capable of acting as a silent partner therefor, binds to said ecdysone response element, and

(iii) a ligand for said modified ecdysone receptor;

said method comprising subjecting said subject to conditions suitable to induce expression of said modified ecdysone receptor.

Inducible promoters contemplated for use in the practice of the present invention are transcription regulatory regions that do not function to transcribe mRNA unless inducing conditions are present. Examples of suitable inducible promoters include DNA sequences corresponding to: the *E. coli* lac operator responsive to IPTG (see Nakamura et al., Cell, 18:1109-1117, 1979); the metallothionein promoter metal-regulatory-elements responsive to heavy-metal (e.g. zinc) induction (see Evans et. al, U.S. Patent No. 4,870,009), the phage T7lac promoter responsive to IPTG (see Studier et al., Meth. Enzymol., 185: 60-89, 1990; and U.S. #4,952,496), the heat-shock promoter, and the like.

In accordance with another embodiment of the present invention, there are provided methods of inducing expression of an exogenous gene in a mammalian subject containing a DNA construct comprising said exogenous gene

under the control of an ecdysone response element, said method comprising introducing into said subject:

a modified ecdysone receptor; and

a ligand for said modified ecdysone receptor,

wherein said receptor, in combination with a ligand therefor, and optionally in the further presence of a receptor capable of acting as a silent partner therefor, binds to said ecdysone response element, activating transcription therefrom.

In accordance with another embodiment of the present invention, there are provided methods for the expression of recombinant products detrimental to a host organism, said method comprising:

transforming suitable host cells with:

(i) a DNA construct encoding said recombinant product under the control of an ecdysone response element, and

(ii) DNA encoding a modified ecdysone receptor;

growing said host cells in suitable media; and

inducing expression of said recombinant product by introducing into said host cells ligand(s) for said modified ecdysone receptor, and optionally a receptor capable of acting as a silent partner for said modified ecdysone receptor.

Recombinant products detrimental to a host organism contemplated for expression in accordance with the present invention include any gene product that functions to confer a toxic effect on the organism. For example, inducible expression of a toxin such as the diptheroid toxin would allow for inducible tissue specific ablation (Ross et al. (1993) *Genes and Development* **7**, 1318-1324). Thus, the numerous gene products that are known to induce apoptosis in cells expressing such products are

contemplated for use herein (see, e.g, Apoptosis, The Molecular Basis of Cell Death, Current Communications In Cell & Molecular Biology, Cold Spring Harbor Laboratory Press, 1991).

Suitable media contemplated for use in the practice of the present invention include any growth and/or maintenance media, in the substantial absence of ligand(s) which, in combination with an invention modified ecdysone receptor, is(are) capable of binding to an ecdysone response element.

In accordance with another embodiment of the present invention, there are provided gene transfer vectors useful for the introduction of invention constructs into suitable host cells. Such gene transfer vectors comprise a transcription regulatory region having a minimal promoter (i.e., a promoter region that does not have an enhancer), and an invention modified ecdysone response element, wherein said regulatory region is operatively associated with DNA containing an exogenous gene, and wherein said modified ecdysone response element is present in multiple copies. The number of copies of response elements can readily be varied by those of skill in the art. For example, transcription regulatory regions can contain from 1 up to about 50 copies of a particular response element, preferably 2 up to about 25 copies, more preferably 3 up to about 10-15 copies, with about 4-6 copies being especially preferred.

Gene transfer vectors (also referred to as "expression vectors") contemplated for use herein are recombinant nucleic acid molecules that are used to transport exogenous nucleic acid into cells for expression and/or replication thereof. Expression vectors may be either circular or linear, and are capable of incorporating a variety of nucleic acid constructs therein. Expression

vectors typically come in the form of a plasmid that, upon introduction into an appropriate host cell, results in expression of the inserted DNA.

As used herein, the phrase "transcription regulatory region" refers to the region of a gene or expression construct that controls the initiation of mRNA transcription. Regulatory regions contemplated for use herein typically comprise at least a minimal promoter in combination with an ecdysone response element. A minimal promoter, when combined with an enhancer region (e.g., a hormone response element), functions to initiate mRNA transcription in response to a ligand/receptor complex. However, transcription will not occur unless the required inducer (ligand) is present.

As used herein, the phrase "operatively associated with" refers to the functional relationship of DNA with regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences. For example, operative linkage of DNA to a promoter refers to the physical and functional relationship between the DNA and promoter such that the transcription of such DNA is initiated from the promoter by an RNA polymerase that specifically recognizes, binds to and transcribes the DNA.

Preferably, the transcription regulatory region further comprises a binding site for an ubiquitous transcription factor. Such a binding site is preferably positioned between the promoter and modified ecdysone response element of the invention. Suitable ubiquitous transcription factors for use herein are well-known in the art and include, for example, Sp1.

Expression vectors suitable for use in the practice of the present invention are well known to those

of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells as well as those that remain episomal and those that integrate into the host cell genome. Expression vectors typically further contain other functionally important nucleic acid sequences, such as expression constructs encoding antibiotic resistance proteins, and the like.

Exemplary eukaryotic expression vectors include eukaryotic constructs, such as the pSV-2 gpt system (Mulligan et al., Nature, 1979, 277:108-114); pBlueSkript (Stratagene, La Jolla, CA), the expression cloning vector described by Genetics Institute (Science, 1985, 228:810-815), and the like. Each of these plasmid vectors are capable of promoting expression of the invention chimeric protein of interest.

Promoters, depending upon the nature of the regulation, may be constitutively or inducibly regulated, or may be tissue-specific (e.g., expressed only in T-cells, endothelial cels, smooth muscle cells, and the like). Exemplary promoters contemplated for use in the practice of the present invention include the SV40 early promoter, the cytomegalovirus (CMV) promoter, the mouse mammary tumor virus (MMTV) steroid-inducible promoter, Moloney murine leukemia virus (MMLV) promoter, elongation factor 1α (EF1α) promoter, albumin promoter, APO A1 promoter, cyclic AMP dependent kinase II (CaMKII) promoter, keratin promoter, CD3 promoter, immunoglobulin light or heavy chain promoters, neurofiliment promoter, neuron specific enolase promoter, L7 promoter, CD2 promoter, myosin light chain kinase promoter, HOX gene promoter, thymidine kinase (TK) promoter, RNA Pol II promoter, MYOD promoter, MYF5 promoter, phophoglycerokinase (PGK) promoter, Stf1 promoter, Low Density Lipoprotein (LDL) promoter,and the like.

Suitable means for introducing (transducing) expression vectors containing nucleic acid constructs according to the invention into host cells to produce transduced recombinant cells (i.e., cells containing recombinant heterologous nucleic acid) are well-known in the art (see, for review, Friedmann, 1989, Science, 244:1275-1281; Mulligan, 1993, Science, 260:926-932, each of which are incorporated herein by reference in their entirety). Exemplary methods of transduction include, e.g., infection employing viral vectors (see, e.g., U.S. Patent 4,405,712 and 4,650,764), calcium phosphate transfection (U.S. Patents 4,399,216 and 4,634,665), dextran sulfate transfection, electroporation, lipofection (see, e.g., U.S. Patents 4,394,448 and 4,619,794), cytofection, particle bead bombardment, and the like. The heterologous nucleic acid can optionally include sequences which allow for its extrachromosomal (i.e., episomal) maintenance, or the heterologous nucleic acid can be donor nucleic acid that integrates into the genome of the host.

In a specific embodiment, said gene transfer vector is a viral vector, preferably a retroviral vector. Retroviral vectors are gene transfer plasmids that have an expression construct encoding an heterologous gene residing between two retroviral LTRs. Retroviral vectors typically contain appropriate packaging signals that enable the retroviral vector, or RNA transcribed using the retroviral vector as a template, to be packaged into a viral virion in an appropriate packaging cell line (see, e.g., U.S. Patent 4,650,764).

Suitable retroviral vectors for use herein are described, for example, in U.S. Patents 5,399,346 and 5,252,479; and in WIPO publications WO 92/07573, WO 90/06997, WO 89/05345, WO 92/05266 and WO 92/14829, incorporated herein by reference, which provide a

description of methods for efficiently introducing nucleic acids into human cells using such retroviral vectors. Other retroviral vectors include, for example, mouse mammary tumor virus vectors (e.g., Shackleford et al., 1988, PNAS, USA, 85:9655-9659), and the like.

Various procedures are also well-known in the art for providing helper cells which produce retroviral vector particles which are essentially free of replicating virus. See, for example, U.S. Patent 4,650,764; Miller, Human Gene Therapy, 1:5-14 (1990); Markowitz, et al., Journal of Virology, 61(4):1120-1124 (1988); Watanabe, et al., Molecular and Cellular Biology, 3(12):2241-2249 (1983); Danos, et al., Proc. Natl. Acad. Sci., 85:6460-6464 (1988); and Bosselman, et al., Molecular and Cellular Biology, 7(5):1797-1806 (1987), which disclose procedures for producing viral vectors and helper cells which minimize the chances for producing a viral vector which includes a replicating virus.

Recombinant retroviruses suitable for carrying out the invention methods are produced employing well-known methods for producing retroviral virions. See, for example, U.S. Patent 4,650,764; Miller, Human Gene Therapy, 1:5-14 (1990); Markowitz, et al., Journal of Virology, 61(4):1120-1124 (1988); Watanabe, et al., Molecular and Cellular Biology, 3(12):2241-2249 (1983); Danos, et al., Proc. Natl. Acad. Sci., 85:6460-6464 (1988); and Bosselman, et al., Molecular and Cellular Biology, 7(5):1797-1806 (1987).

In accordance with another embodiment of the present invention, there are provided recombinant cells containing a nucleic acid encoding modified ecdysone receptor(s) according to the invention. Exemplary eukaryotic cells for introducing expression vectors according to the invention include, e.g., CV-1 cells, P19

cells and NT2/D1 cells (which are derived from human embryo carcinomas), COS cells, mouse L cells, Chinese hamster ovary (CHO) cells, primary human fibroblast cells, human embryonic kidney cells, African green monkey cells, HEK 293 (ATCC accession #CRL 1573; U.S. Patent No. 5,024,939), Ltk^- cells (ATCC accession #CCL1.3), COS-7 cells (ATCC under accession #CRL 1651), DG44 cells ($dhfr^-$ CHO cells; see, e.g., Urlaub et al. (1986) Cell. Molec. Genet. 12: 555), cultured primary tissues, cultured tumor cells, and the like. Presently preferred cells include CV-1 and 293 cells.

In accordance with another embodiment of the present invention, there is provided a transgenic mammal containing a nucleic acid encoding an invention modified ecdysone receptor. As used herein, the phrase "transgenic mammal" refers to a mammal that contains one or more inheritable expression constructs containing a recombinant modified ecdysone receptor transgene and/or an exogenous gene under the transcription control of an invention modified ecdysone response element. Preferably, an invention transgenic mammal also contains one or more inheritable expression constructs containing a member of the steroid/thyroid hormone superfamily of receptors that functions as a silent partner for modified ecdysone receptor (e.g., RXR).

Methods of making transgenic mammals using a particular nucleic acid construct are well-known in the art. When preparing invention transgenic animals, it is preferred that two transgenic lines are generated. The first line will express, for example, RXR and a modified EcR (e.g., VpEcR). Tissue specificity is conferred by the selection of tissue-specific promoters (e.g., T-cell specific) that will then direct the expression of the receptors. A second line contains an ecdysone responsive promoter controlling the expression of an exogenous cDNA.

In a preferred embodiment of the present invention, an invention transgenic mammal contains one or more expression constructs containing nucleic acid encoding a modified ecdysone receptor, exogenous RXR, and an exogenous gene under the transcription control of an invention modified ecdysone response element. It has been found that in transgenic mice containing an ecdysone inducible promoter (i.e., an invention modified ecdysone response element) and expressing a modified ecdysone receptor and RXR, muristerone treatment can activate gene expression. Thus, with tissue specific expression of the modified ecdysone receptor and RXR and timely hormone treatment, inducible gene expression can be achieved with spatial, dosage, and temporal specificity.

In accordance with another embodiment of the present invention, there are provided methods for inducing expression of an exogenous gene in a transgenic mammal containing a modified ecdysone receptor according to the invention, said method comprising:

introducing into said mammal a DNA construct encoding an exogenous gene under the transcription control of an ecdysone response element responsive to said modified ecdysone receptor; and

administering to said mammal an amount of ligand for said modified ecdysone receptor effective to induce expression of said exogenous gene.

As discussed hereinbefore, the modified ecdysone receptor forms a homodimer, or optionally a heterodimer in the presence of a silent partner of the steroid/thyroid hormone superfamily of receptors, and functions to activate transcription from an expression vector having a response

element responsive to the particular homodimer or heterodimer formed.

In accordance with another embodiment of the present invention, there are provided methods for the induction of two different genes in a mammalian subject comprising: activating a first exogenous gene employing the invention ecdysone inducible system; and activating a second gene using a tetracycline inducible system. The invention method for inducing two different genes is particularly advantagous because it permits the temporal, spatial, and dosage specific control of two exogenous genes.

The tetracycline inducible system is well-known in the art (see, e.g, Gossen et al. (1992) *Proc. Natl. Acad. Sci.* **89,** 5547-5551; Gossen et al.(1993) *TIBS* **18,** 471-475; Furth et al. (1994) *Proc. Natl. Acad. Sci.* **91,** 9302-9306; and Shockett et al. (1995) *Proc. Natl. Acad. Sci.* **92,** 6522-6526).

All U.S. and Foreign Patent publications, textbooks, and journal publications referred to herein are hereby expressly incorporated by reference in their entirety. The invention will now be described in greater detail by reference to the following non-limiting examples.

Example 1

Preparation of modified ecdysone receptors

Plasmid preparation:

The plasmids CMX-EcR, CMX-USP, CMX-FXR, CMX-hRXRa, EcREx5-ΔMTV-Luc, CMX-GEcR, MMTV-luc, and CMX-GR have been previously described (Yao, et al., *Nature* **366**:476-479 (1993) and Forman, et al. *Cell* **81**:687-693 (1995)).

The plasmid CMX-VpEcR was constructed by ligation of an EcoRI fragment of psk-EcR and CMX-Vp16.

The plasmid CMX-VgEcR was generated by site-directed mutagenesis of CMX-VpEcR using the Transformer Mutagenesis Kit (Clontech) and the mutagenic Oligonucleotide (SEQ ID NO:14):

5'-TACAACGCCCTCACCTGTGGATCCTGCAAGGTGTTTCTTTCGACGCAGC-3'.

Mutagenesis of VpEcR to VgEcR altered the P-box region of the DNA binding domain of ecdysone receptor to resemble that of GR (Umesono and Evans, *Cell* **57**:1139-1146 (1989)). The following amino acids in the DNA-binding domain of the ecdysone receptor were altered: E282G, G283S, and G286V (E=glutamate, G=glycine, S=serine, V=valine).

The reporter construct EcREx4-ΔHSP-ß-gal was constructed by oligomerizing two annealed oligonucleotides containing the HSP-EcRE (Yao, et al., *Nature* **366**:476-479 (1993)).

EcREx4-Sp1x3-ΔHSP-ßgal was constructed by ligating the following annealed oligonucleotides into the Asp718 site of EcREx4-HSP-ß-gal (SEQ ID NO:15):

5'-GTACTCCCGGGGCGGGGCTATGCGGGGCGGGGCTAATCGCTAGGGGCGGGGCA-3'

and (SEQ ID NO:16):

5'-GTACTGCCCCGCCCCTAGCGATTAGCCCCGCCCCGCATAGCCCCGCCC
CGGGA-3'.

ΔHSP is a minimal promoter derived from the *Drosophila* heat shock promoter with its enhancers deleted.

To generate the construct E/GREx4-ΔMTV-Luc, the following oligonucleotides (SEQ ID NO:17):

5'-AGCTCGATGGACAAGTGCATTGTTCTTTGCTGAA-3';

and (SEQ ID NO:18):

5'-AGCTTTCAGCAAGAGAACAATGCACTTGTCCATCG-3',

were annealed, multimerized, and ligated into the HindIII site of ΔMTV-Luc. The resulting reporter contained 4 copies of the invention modified ecdysone response element E/GRE.

To produce the plasmid pRC-ESHß, a BglII/(XhoI) fragment containing EcREx4-Sp1x3-ΔHSP-ß-gal was subcloned into BglII/(NotI) digested pRC-CMV (Invitrogen, San Diego, CA), which contains a neomycin resistance gene.

Cell Culture and Transient Transfections:

CV-1 cells were maintained in DMEM supplemented with 10% Fetal Bovine Serum. Transient transfections were performed using DOTAP transfection reagent (Boehringer-Mannheim). Transfections using ß-galactosidase as the reporter were assayed either by Galactolight luminescent assay (Tropix, Bedford, MA) or by standard liquid ONPG assay (Sigma, St. Louis, MO). The values were normalized by co-transfection of CMX-luciferase. Transfections using luciferase as the reporter were assayed by standard techniques using luciferin and ATP. These values were normalized by co-transfection of CMX-ß-galactosidase. Hormone treated cells were treated with ethanol, 50 μM Juvenile Hormone III (Sigma), 1μM

muristerone A (Zambon, Bresso, IT), or 1μM dexamethasone (Sigma) unless otherwise noted.

To maximize the sensitivity of the invention ecdysone inducible system, modifications of the ecdysone receptor were made. The N-terminal transactivation domain of the ecdysone receptor was replaced by the corresponding domain of the glucocorticoid receptor (GR), resulting in the modified ecdysone receptor GEcR (See Figure 1D). CV-1 cells were transfected with the plasmid CMX-GEcR encoding the modified ecdysone receptor as discussed above. After transfection, cells were either treated with ethanol or 1μM muristerone A. This hybrid modified ecdysone receptor boosted muristerone responsiveness from 3- to 11-fold in a transient transfection assay (Fig. 1A). Replacement of the natural heterodimeric partner for the ecdysone receptor, USP, by its mammalian homologue, the retinoid X receptor (RXR), produced a more potent ligand dependent heterodimer, providing a 34 fold induction (Fig. 1A).

A more potent heterodimer, however, was obained by combining RXR and VpEcR, an N-terminal truncation of the ecdysone receptor attached to the VP16 activation domain, resulting in a 212 fold induction (Fig. 1A and 1D). Different from most nuclear receptor/VP16 fusion proteins which exhibit high constitutive activity, VpEcR generates ligand dependent superinduction while maintaining a very low basal activity (Underhill et al., *Mol. Encod.* **8**:274-285 (1994) and Perlmann et al., *Genes & Devel.* **7**:1411-1422 (1993)).

In addition, the reporter vector was also modified by inserting consensus binding sites for the ubiquitous transcription factor Sp1 between the minimal promoter and the ecdysone response elements (Kamine et al., *Proc. Natl. Acad. Sci.* **88**:8510-8514 (1991) and Strahle te

al., *EMBO* **7**:3389-3395 (1988)). The addition of Sp1 sites to the ecdysone responsive promoter increases the absolute activity 5-fold (Fig. 1A).

Example 2
Construction of a novel ecdysone response element

Although no mammalian transcription factors have been shown to have a natural enhancer element like the ecdysone response element, which is composed of two inverted half-sites of the sequence AGGTCA spaced by one nucleotide, it is difficult to preclude such a possibility. The recently cloned farnesoid X receptor (FXR) can very weakly activate certain synthetic promoters containing an ecdysone response element in response to extremely high concentrations of farnesoids (Forman et al., *Cell* **81**:687-693 (1995)).

In FXR containing cells and in transgenic mice, activation of gene expression by endogenous receptors would create undesirable background levels of reporter protein. To circumvent this potential problem, the DNA binding specificity of VpEcR was altered to mimic that of GR, which binds as a homodimer to an inverted repeat of the sequence AGAACA, spaced by three nucleotides. This altered binding specificity was achieved by mutating three amino acid residues of VpEcR in the P-box of the DNA binding domain, a region previously shown to be essential for DNA sequence recognition (Umesono and Evans, *Cell* **57**:1139-1146 (1989)). This new hybrid modified ecdysone receptor is referred herein as VgEcR and is responsive to a new hybrid respone element referred to herein as the E/GRE (SEQ ID NO:12), which contains two different half-site motifs, RGBNNM and RGNNCA, spaced by one nucleotide (Fig. 1B). This new response element is a hybrid between the glucocorticoid response element (GRE) and that of type II nuclear receptors like RXR, EcR, retinoic acid receptor (RAR),

thyroid hormone receptor (T3R), etc. Although FXR can activate a promoter containing the wild type ecdysone response element, it cannot activate one containing the E/GRE (Fig 1B; note log scale). The E/GRE reporter is not activated by GR nor does VgEcR activate a dexamethasone responsive promoter (Fig 1C).

Example 3

Assay for Ecdysone responsiveness in stable cell lines

Stable cell lines were generated containing the modified ecdysone receptor VpEcR, a heterodimeric partner (RXR), and an ecdysone inducible reporter (Figure 2). 293 cells were transfected with the following linearized plasmids, pRC-ESHß, EcREx5-ΔMTV-Luc, CMX-VpEcR, and CMX-hRXRa. The following day, the cells were split 1:10 and were allowed to recover one day prior to selection with 1mg/ml G418 (GIBCO). After 14 days of selection, 14 individual clones were isolated and grown separately in the presence of 0.5mg/ml G418. Of 14 G418 resistant clones, 10 demonstrated differing degrees of muristerone responsiveness.

One of these cell lines, N13, was grown in the presence or absence of 1μM muristerone for 20 hours. Cell lysates were then assayed for ß-galactosidase and luciferase activities as described in Example 1. X-gal staining was performed on the stable cell lines. Cells were fixed briefly with 10% formaldehyde in PBS and then stained with X-Gal (Molecular Probes, Eugene, OR) for 2 to 6 hours. After 24 hours of treatment with 1μM muristerone, 100% of the cells turned dark blue after 3 hours of staining. Thus, mammalian cells containing the modified ecdysone receptor VpEcR, a heterodimeric partner (RXR), and a reporter gene construct regulated by a modified ecdysone

response element, function to efficiently express an exogenous gene in response to a ligand, e.g., ecdysone.

A dose-response assay was conducted by growing N13 cells with varying concentrations of muristerone for 36 hours and then assaying for ß-galactosidase activity (using the well-known ONPG assay), or the cells were assayed for luciferase activity. Dose response curves of stably integrated ß-galactosidase and luciferase reporters in N13 cells revealed that inducibility approaching 3 orders of magnitude can be achieved at a final concentration 10μM muristerone (Figure 3A). One-hundred fold induction was achieved by muristerone concentrations as low as 100nM (Figure 3A).

Finally, the kinetics of muristerone mediated induction was measured. N13 cells were grown in separate wells in the presence of 1μM muristerone, harvested at varying times, and assayed for luciferase activity. Inductions of 100-fold in 3 hrs., 1000 fold in 8 hrs., and maximal effects of 20,000 fold after 20 hours of treatment were observed (Figure 3B). Similar results were observed in stable lines containing CMX-VgEcR and the E/GRE reporters.

Example 4

Bioavailability and activity of muristerone

In order to use muristerone as a potential hormone in mice, its toxicity and bioavailability was examined. For toxicity studies, adult mice were injected intraperitonealy with 20mg of muristerone A suspended in sesame oil. The mice were then observed for approximately two months. For teratogenic studies, pregnant mice were injected with 20mg of muristerone A suspended in sesame oil and both the mother and pups were observed for three

months. The results indicate that muristerone maintains its activity when injected into mice, and that it is neither toxic, teratogenic, nor inactivated by serum binding proteins. In addition to the inert qualities of muristerone (an ecdysone), overexpression of VpEcR and RXR appears not to be toxic.

For muristerone bioavailability studies, adult mice were injected intraperitoneally with sesame oil with or without 10mg of muristerone, and were subsequently sacrificed for serum collection. After twelve hours, blood was drawn from the mice, and the serum was isolated by brief centrifugation of the whole blood. In order to conduct transfection assays to test for muristerone activity, serum from sesame oil injected mice was divided, and half was supplemented with muristerone to a final concentration of 10μM. The three batches of mouse serum were diluted 1:10 in DMEM and placed onto CV-1 cells transfected with CMX-GEcR, CMX-hRXRa, and EcREx5-DMTV-Luc.

The results are shown in Figure 4 and indicate that serum from muristerone treated mice yielded a luciferase activity comparable to that seen from untreated mouse serum supplemented with 1μM muristerone. The results indicate that single-site injected material should be widely circulated, and that there is little or no blunting of activity due to association with serum proteins.

Example 5

Muristerone dependent gene expression in transgenic mice

To produce transgenic mice, the following DNA constructs were prepared and subsequently injected into fertilized eggs: CD3-VpEcR, CD3-RXR, ESHß (Lee et al., *J. Exp. Med.* **175**:1013-1025 (1992)). Two separate lines of transgenic mice were generated harboring either an ecdysone

inducible reporter, ESHß, or a T-cell specific expression construct of VpEcR and RXR, respectively. The former are referred to as reporter mice, the latter are referred to as receptor mice, and double transgenic mice are referred to as receptor/reporter mice. Constructs CD3-VpEcR and CD3-RXR were mixed and coinjected, while ESHß was injected alone. Primary genotyping was performed by Southern blot analysis and the transmission of transgenic mice was monitored by dot blot analysis. Receptor mice were analyzed for VpEcR and RXR expression by Northern blot analysis of RNA collected from these mice. For Northern blot analysis, 15μg of total RNA obtained from the thymus, and various tissues as a control, was run on a denaturing gel and blotted onto a nitrocellulose membrane. The blot was probed with a radiolabeled ß-gal-specific probe and exposed on film for 2 days. These receptor mice were healthy, fertile, and appeared normal by visual inspection.

In addition, the transgene was transferred to the offspring as expected by Mendelian genetics. This data suggests that overexpression of VpEcR and RXR in T-cells is not toxic.

Receptor expressing mice were bred with reporter mice (containing ESHß) to produce double transgenic receptor/reporter mice. Adult receptor/reporter transgenic mice (genotype=CD3-VpEcR; CD3-RXR; and ESHβ) were injected intraperitonealy with sesame oil with or without 10mg of muristerone. Subsequently, a Northern blot analysis was performed on the double transgenic lines using RNA isolated 48 hours after treatment from various tissues including the thymus, brain and liver, to test for the specific induction of an ecdysone inducible promoter. The probe used was specific to the activity of the ecdysone inducible promoter. The autoradiograph was exposed for 36 hrs. The results of the Northern analysis indicate that muristerone treatment of the transgenic mouse containing a T-cell

specific expression construct of VpEcR and RXR, and the ecdysone inducible reporter ESHß, caused a significant induction from an ecdysone inducible promoter in the thymus, while low basal activity is observed in its absence.

Example 6

Assay for Ponasterone responsiveness

A dose-response assay was conducted as described in Example 3, by growing N13 cells with varying concentrations of muristerone or ponasterone A for 36 hours and then assaying for ß-galactosidase activity (using the well-known ONPG assay), or the cells were assayed for luciferase activity. Dose response curves of stably integrated ß-galactosidase and luciferase reporters in N13 cells revealed that inducibility exceeding 3 orders of magnitude can be achieved with both ligands at final concentrations of about 10^{-4} (see Figure 5).

While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: HORMONE-MEDIATED METHODS FOR MODULATING EXPRESSION OF EXOGENOUS GENES IN MAMMALIAN SYSTEMS, AND PRODUCTS RELATED THERETO

(iii) NUMBER OF SEQUENCES: 18

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/974,530
(B) FILING DATE: November 19, 1997
(C) CLASSIFICATION:

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(C) REFERENCE/DOCKET NUMBER: SALK1520-2

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Cys Xaa Xaa Cys Xaa Xaa Asp Xaa Ala Xaa Gly Xaa Tyr Xaa Xaa Xaa
1 5 10 15

Xaa Cys Xaa Xaa Cys Lys Xaa Phe Phe Xaa Arg Xaa Xaa Xaa Xaa Xaa
20 25 30

Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys
35 40 45

Xaa Xaa Xaa Lys Xaa Xaa Arg Xaa Xaa Cys Xaa Xaa Cys Arg Xaa Xaa
50 55 60

Lys Cys Xaa Xaa Xaa Gly Met
65 70

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Glu Gly Cys Lys Gly
1 5

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Gly Ser Cys Lys Val
1 5

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2241 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..2241
(D) OTHER INFORMATION: /product= "VgEcR"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATG GCC CCC CCG ACC GAT GTC AGC CTG GGG GAC GAG CTC CAC TTA GAC 48
Met Ala Pro Pro Thr Asp Val Ser Leu Gly Asp Glu Leu His Leu Asp
1 5 10 15

GGC GAG GAC GTG GCG ATG GCG CAT GCC GAC GCG CTA GAC GAT TTC GAT 96
Gly Glu Asp Val Ala Met Ala His Ala Asp Ala Leu Asp Asp Phe Asp
20 25 30

CTG GAC ATG TTG GGG GAC GGG GAT TCC CCG GGT CCG GGA TTT ACC CCC 144
Leu Asp Met Leu Gly Asp Gly Asp Ser Pro Gly Pro Gly Phe Thr Pro
35 40 45

CAC GAC TCC GCC CCC TAC GGC GCT CTG GAT ATG GCC GAC TTC GAG TTT 192
His Asp Ser Ala Pro Tyr Gly Ala Leu Asp Met Ala Asp Phe Glu Phe
50 55 60

GAG CAG ATG TTT ACC GAT GCC CTT GGA ATT GAC GAG TAC GGT GGG AAG 240
Glu Gln Met Phe Thr Asp Ala Leu Gly Ile Asp Glu Tyr Gly Gly Lys
65 70 75 80

CTT CTA GGT ACC TCT AGA AGG ATA TCG AAT TCT ATA TCT TCA GGT CGC 288
Leu Leu Gly Thr Ser Arg Arg Ile Ser Asn Ser Ile Ser Ser Gly Arg
85 90 95

GAT GAT CTC TCG CCT TCG AGC AGC TTG AAC GGA TAC TCG GCG AAC GAA 336
Asp Asp Leu Ser Pro Ser Ser Ser Leu Asn Gly Tyr Ser Ala Asn Glu
100 105 110

AGC TGC GAT GCG AAG AAG AGC AAG AAG GGA CCT GCG CCA CGG GTG CAA 384
Ser Cys Asp Ala Lys Lys Ser Lys Lys Gly Pro Ala Pro Arg Val Gln
115 120 125

GAG GAG CTG TGC CTG GTT TGC GGC GAC AGG GCC TCC GGC TAC CAC TAC 432
Glu Glu Leu Cys Leu Val Cys Gly Asp Arg Ala Ser Gly Tyr His Tyr
130 135 140

AAC GCC CTC ACC TGT GGA TCC TGC AAG GTG TTC TTT CGA CGC AGC GTT 480
Asn Ala Leu Thr Cys Gly Ser Cys Lys Val Phe Phe Arg Arg Ser Val
145 150 155 160

ACG AAG AGC GCC GTC TAC TGC TGC AAG TTC GGG CGC GCC TGC GAA ATG 528
Thr Lys Ser Ala Val Tyr Cys Cys Lys Phe Gly Arg Ala Cys Glu Met
165 170 175

GAC ATG TAC ATG AGG CGA AAG TGT CAG GAG TGC CGC CTG AAA AAG TGC 576
Asp Met Tyr Met Arg Arg Lys Cys Gln Glu Cys Arg Leu Lys Lys Cys
180 185 190

CTG GCC GTG GGT ATG CGG CCG GAA TGC GTC GTC CCG GAG AAC CAA TGT 624
Leu Ala Val Gly Met Arg Pro Glu Cys Val Val Pro Glu Asn Gln Cys
195 200 205

GCG ATG AAG CGG CGC GAA AAG AAG GCC CAG AAG GAG AAG GAC AAA ATG 672
Ala Met Lys Arg Arg Glu Lys Lys Ala Gln Lys Glu Lys Asp Lys Met
210 215 220

ACC ACT TCG CCG AGC TCT CAG CAT GGC GGC AAT GGC AGC TTG GCC TCT 720
Thr Thr Ser Pro Ser Ser Gln His Gly Gly Asn Gly Ser Leu Ala Ser
225 230 235 240

GGT GGC GGC CAA GAC TTT GTT AAG AAG GAG ATT CTT GAC CTT ATG ACA 768
Gly Gly Gly Gln Asp Phe Val Lys Lys Glu Ile Leu Asp Leu Met Thr
245 250 255

TGC GAG CCG CCC CAG CAT GCC ACT ATT CCG CTA CTA CCT GAT GAA ATA 816
Cys Glu Pro Pro Gln His Ala Thr Ile Pro Leu Leu Pro Asp Glu Ile
260 265 270

TTG GCC AAG TGT CAA GCG CGC AAT ATA CCT TCC TTA ACG TAC AAT CAG 864
Leu Ala Lys Cys Gln Ala Arg Asn Ile Pro Ser Leu Thr Tyr Asn Gln
275 280 285

TTG GCC GTT ATA TAC AAG TTA ATT TGG TAC CAG GAT GGC TAT GAG CAG 912
Leu Ala Val Ile Tyr Lys Leu Ile Trp Tyr Gln Asp Gly Tyr Glu Gln
290 295 300

CCA TCT GAA GAG GAT CTC AGG CGT ATA ATG AGT CAA CCC GAT GAG AAC 960
Pro Ser Glu Glu Asp Leu Arg Arg Ile Met Ser Gln Pro Asp Glu Asn
305 310 315 320

GAG AGC CAA ACG GAC GTC AGC TTT CGG CAT ATA ACC GAG ATA ACC ATA 1008
Glu Ser Gln Thr Asp Val Ser Phe Arg His Ile Thr Glu Ile Thr Ile
325 330 335

CTC ACG GTC CAG TTG ATT GTT GAG TTT GCT AAA GGT CTA CCA GCG TTT 1056
Leu Thr Val Gln Leu Ile Val Glu Phe Ala Lys Gly Leu Pro Ala Phe
340 345 350

ACA AAG ATA CCC CAG GAG GAC CAG ATC ACG TTA CTA AAG GCC TGC TCG 1104
Thr Lys Ile Pro Gln Glu Asp Gln Ile Thr Leu Leu Lys Ala Cys Ser
355 360 365

TCG GAG GTG ATG ATG CTG CGT ATG GCA CGA CGC TAT GAC CAC AGC TCG 1152
Ser Glu Val Met Met Leu Arg Met Ala Arg Arg Tyr Asp His Ser Ser
370 375 380

GAC TCA ATA TTC TTC GCG AAT AAT AGA TCA TAT ACG CGG GAT TCT TAC 1200
Asp Ser Ile Phe Phe Ala Asn Asn Arg Ser Tyr Thr Arg Asp Ser Tyr
385 390 395 400

AAA ATG GCC GGA ATG GCT GAT AAC ATT GAA GAC CTG CTG CAT TTC TGC 1248
Lys Met Ala Gly Met Ala Asp Asn Ile Glu Asp Leu Leu His Phe Cys
405 410 415

CGC CAA ATG TTC TCG ATG AAG GTG GAC AAC GTC GAA TAC GCG CTT CTC 1296
Arg Gln Met Phe Ser Met Lys Val Asp Asn Val Glu Tyr Ala Leu Leu
420 425 430

ACT GCC ATT GTG ATC TTC TCG GAC CGG CCG GGC CTG GAG AAG GCC CAA 1344
Thr Ala Ile Val Ile Phe Ser Asp Arg Pro Gly Leu Glu Lys Ala Gln
435 440 445

CTA GTC GAA GCG ATC CAG AGC TAC TAC ATC GAC ACG CTA CGC ATT TAT 1392
Leu Val Glu Ala Ile Gln Ser Tyr Tyr Ile Asp Thr Leu Arg Ile Tyr
450 455 460

ATA CTC AAC CGC CAC TGC GGC GAC TCA ATG AGC CTC GTC TTC TAC GCA 1440
Ile Leu Asn Arg His Cys Gly Asp Ser Met Ser Leu Val Phe Tyr Ala
465 470 475 480

AAG CTG CTC TCG ATC CTC ACC GAG CTG CGT ACG CTG GGC AAC CAG AAC 1488
Lys Leu Leu Ser Ile Leu Thr Glu Leu Arg Thr Leu Gly Asn Gln Asn
485 490 495

GCC GAG ATG TGT TTC TCA CTA AAG CTC AAA AAC CGC AAA CTG CCC AAG 1536
Ala Glu Met Cys Phe Ser Leu Lys Leu Lys Asn Arg Lys Leu Pro Lys
500 505 510

TTC CTC GAG GAG ATC TGG GAC GTT CAT GCC ATC CCG CCA TCG GTC CAG 1584
Phe Leu Glu Glu Ile Trp Asp Val His Ala Ile Pro Pro Ser Val Gln
515 520 525

TCG CAC CTT CAG ATT ACC CAG GAG GAG AAC GAG CGT CTC GAG CGG GCT 1632
Ser His Leu Gln Ile Thr Gln Glu Glu Asn Glu Arg Leu Glu Arg Ala
530 535 540

GAG CGT ATG CGG GCA TCG GTT GGG GGC GCC ATT ACC GCC GGC ATT GAT 1680
Glu Arg Met Arg Ala Ser Val Gly Gly Ala Ile Thr Ala Gly Ile Asp
545 550 555 560

TGC GAC TCT GCC TCC ACT TCG GCG GCG GCA GCC GCG GCC CAG CAT CAG 1728
Cys Asp Ser Ala Ser Thr Ser Ala Ala Ala Ala Ala Ala Gln His Gln
565 570 575

CCT CAG CCT CAG CCC CAG CCC CAA CCC TCC TCC CTG ACC CAG AAC GAT 1776
Pro Gln Pro Gln Pro Gln Pro Gln Pro Ser Ser Leu Thr Gln Asn Asp
580 585 590

TCC CAG CAC CAG ACA CAG CCG CAG CTA CAA CCT CAG CTA CCA CCT CAG 1824
Ser Gln His Gln Thr Gln Pro Gln Leu Gln Pro Gln Leu Pro Pro Gln
595 600 605

CTG CAA GGT CAA CTG CAA CCC CAG CTC CAA CCA CAG CTT CAG ACG CAA 1872
Leu Gln Gly Gln Leu Gln Pro Gln Leu Gln Pro Gln Leu Gln Thr Gln
610 615 620

CTC CAG CCA CAG ATT CAA CCA CAG CCA CAG CTC CTT CCC GTC TCC GCT 1920
Leu Gln Pro Gln Ile Gln Pro Gln Pro Gln Leu Leu Pro Val Ser Ala
625 630 635 640

CCC GTG CCC GCC TCC GTA ACC GCA CCT GGT TCC TTG TCC GCG GTC AGT 1968
Pro Val Pro Ala Ser Val Thr Ala Pro Gly Ser Leu Ser Ala Val Ser
645 650 655

ACG AGC AGC GAA TAC ATG GGC GGA AGT GCG GCC ATA GGA CCC ATC ACG 2016
Thr Ser Ser Glu Tyr Met Gly Gly Ser Ala Ala Ile Gly Pro Ile Thr
660 665 670

CCG GCA ACC ACC AGC AGT ATC ACG GCT GCC GTT ACC GCT AGC TCC ACC 2064
Pro Ala Thr Thr Ser Ser Ile Thr Ala Ala Val Thr Ala Ser Ser Thr
675 680 685

ACA TCA GCG GTA CCG ATG GGC AAC GGA GTT GGA GTC GGT GTT GGG GTG 2112
Thr Ser Ala Val Pro Met Gly Asn Gly Val Gly Val Gly Val Gly Val
690 695 700

GGC GGC AAC GTC AGC ATG TAT GCG AAC GCC CAG ACG GCG ATG GCC TTG 2160
Gly Gly Asn Val Ser Met Tyr Ala Asn Ala Gln Thr Ala Met Ala Leu
705 710 715 720

ATG GGT GTA GCC CTG CAT TCG CAC CAA GAG CAG CTT ATC GGG GGA GTG 2208
Met Gly Val Ala Leu His Ser His Gln Glu Gln Leu Ile Gly Gly Val
725 730 735

GCG GTT AAG TCG GAG CAC TCG ACG ACT GCA TAG 2241
Ala Val Lys Ser Glu His Ser Thr Thr Ala
740 745

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 746 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Ala Pro Pro Thr Asp Val Ser Leu Gly Asp Glu Leu His Leu Asp
1 5 10 15

Gly Glu Asp Val Ala Met Ala His Ala Asp Ala Leu Asp Asp Phe Asp
20 25 30

Leu Asp Met Leu Gly Asp Gly Asp Ser Pro Gly Pro Gly Phe Thr Pro
35 40 45

His Asp Ser Ala Pro Tyr Gly Ala Leu Asp Met Ala Asp Phe Glu Phe
50 55 60

Glu Gln Met Phe Thr Asp Ala Leu Gly Ile Asp Glu Tyr Gly Gly Lys
65 70 75 80

Leu Leu Gly Thr Ser Arg Arg Ile Ser Asn Ser Ile Ser Ser Gly Arg
85 90 95

Asp Asp Leu Ser Pro Ser Ser Ser Leu Asn Gly Tyr Ser Ala Asn Glu
100 105 110

Ser Cys Asp Ala Lys Lys Ser Lys Lys Gly Pro Ala Pro Arg Val Gln
115 120 125

Glu Glu Leu Cys Leu Val Cys Gly Asp Arg Ala Ser Gly Tyr His Tyr
130 135 140

Asn Ala Leu Thr Cys Gly Ser Cys Lys Val Phe Phe Arg Arg Ser Val
145 150 155 160

Thr Lys Ser Ala Val Tyr Cys Cys Lys Phe Gly Arg Ala Cys Glu Met
165 170 175

Asp Met Tyr Met Arg Arg Lys Cys Gln Glu Cys Arg Leu Lys Lys Cys
180 185 190

Leu Ala Val Gly Met Arg Pro Glu Cys Val Val Pro Glu Asn Gln Cys
195 200 205

Ala Met Lys Arg Arg Glu Lys Lys Ala Gln Lys Glu Lys Asp Lys Met
210 215 220

Thr Thr Ser Pro Ser Ser Gln His Gly Gly Asn Gly Ser Leu Ala Ser
225 230 235 240

Gly Gly Gly Gln Asp Phe Val Lys Lys Glu Ile Leu Asp Leu Met Thr
245 250 255

Cys Glu Pro Pro Gln His Ala Thr Ile Pro Leu Leu Pro Asp Glu Ile
260 265 270

Leu Ala Lys Cys Gln Ala Arg Asn Ile Pro Ser Leu Thr Tyr Asn Gln
275 280 285

Leu Ala Val Ile Tyr Lys Leu Ile Trp Tyr Gln Asp Gly Tyr Glu Gln
290 295 300

Pro Ser Glu Glu Asp Leu Arg Arg Ile Met Ser Gln Pro Asp Glu Asn
305 310 315 320

Glu Ser Gln Thr Asp Val Ser Phe Arg His Ile Thr Glu Ile Thr Ile
325 330 335

Leu Thr Val Gln Leu Ile Val Glu Phe Ala Lys Gly Leu Pro Ala Phe
340 345 350

Thr Lys Ile Pro Gln Glu Asp Gln Ile Thr Leu Leu Lys Ala Cys Ser
355 360 365

Ser Glu Val Met Met Leu Arg Met Ala Arg Arg Tyr Asp His Ser Ser
370 375 380

Asp Ser Ile Phe Phe Ala Asn Asn Arg Ser Tyr Thr Arg Asp Ser Tyr
385 390 395 400

Lys Met Ala Gly Met Ala Asp Asn Ile Glu Asp Leu Leu His Phe Cys
405 410 415

Arg Gln Met Phe Ser Met Lys Val Asp Asn Val Glu Tyr Ala Leu Leu
420 425 430

Thr Ala Ile Val Ile Phe Ser Asp Arg Pro Gly Leu Glu Lys Ala Gln
435 440 445

Leu Val Glu Ala Ile Gln Ser Tyr Tyr Ile Asp Thr Leu Arg Ile Tyr
450 455 460

Ile Leu Asn Arg His Cys Gly Asp Ser Met Ser Leu Val Phe Tyr Ala
465 470 475 480

Lys Leu Leu Ser Ile Leu Thr Glu Leu Arg Thr Leu Gly Asn Gln Asn
485 490 495

Ala Glu Met Cys Phe Ser Leu Lys Leu Lys Asn Arg Lys Leu Pro Lys
500 505 510

Phe Leu Glu Glu Ile Trp Asp Val His Ala Ile Pro Pro Ser Val Gln
515 520 525

Ser His Leu Gln Ile Thr Gln Glu Glu Asn Glu Arg Leu Glu Arg Ala
530 535 540

Glu Arg Met Arg Ala Ser Val Gly Gly Ala Ile Thr Ala Gly Ile Asp
545 550 555 560

Cys Asp Ser Ala Ser Thr Ser Ala Ala Ala Ala Ala Ala Gln His Gln
565 570 575

Pro Gln Pro Gln Pro Gln Pro Gln Pro Ser Ser Leu Thr Gln Asn Asp
580 585 590

Ser Gln His Gln Thr Gln Pro Gln Leu Gln Pro Gln Leu Pro Pro Gln

595 600 605

Leu Gln Gly Gln Leu Gln Pro Gln Leu Gln Pro Gln Leu Gln Thr Gln
610 615 620

Leu Gln Pro Gln Ile Gln Pro Gln Pro Gln Leu Leu Pro Val Ser Ala
625 630 635 640

Pro Val Pro Ala Ser Val Thr Ala Pro Gly Ser Leu Ser Ala Val Ser
645 650 655

Thr Ser Ser Glu Tyr Met Gly Gly Ser Ala Ala Ile Gly Pro Ile Thr
660 665 670

Pro Ala Thr Thr Ser Ser Ile Thr Ala Ala Val Thr Ala Ser Ser Thr
675 680 685

Thr Ser Ala Val Pro Met Gly Asn Gly Val Gly Val Gly Val Gly Val
690 695 700

Gly Gly Asn Val Ser Met Tyr Ala Asn Ala Gln Thr Ala Met Ala Leu
705 710 715 720

Met Gly Val Ala Leu His Ser His Gln Glu Gln Leu Ile Gly Gly Val
725 730 735

Ala Val Lys Ser Glu His Ser Thr Thr Ala
740 745

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2241 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..2241
(D) OTHER INFORMATION: /product= "VpEcR"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATG GCC CCC CCG ACC GAT GTC AGC CTG GGG GAC GAG CTC CAC TTA GAC 48
Met Ala Pro Pro Thr Asp Val Ser Leu Gly Asp Glu Leu His Leu Asp
1 5 10 15

GGC GAG GAC GTG GCG ATG GCG CAT GCC GAC GCG CTA GAC GAT TTC GAT 96
Gly Glu Asp Val Ala Met Ala His Ala Asp Ala Leu Asp Asp Phe Asp
20 25 30

CTG GAC ATG TTG GGG GAC GGG GAT TCC CCG GGT CCG GGA TTT ACC CCC 144
Leu Asp Met Leu Gly Asp Gly Asp Ser Pro Gly Pro Gly Phe Thr Pro
35 40 45

CAC GAC TCC GCC CCC TAC GGC GCT CTG GAT ATG GCC GAC TTC GAG TTT 192
His Asp Ser Ala Pro Tyr Gly Ala Leu Asp Met Ala Asp Phe Glu Phe
50 55 60

GAG CAG ATG TTT ACC GAT GCC CTT GGA ATT GAC GAG TAC GGT GGG AAG 240
Glu Gln Met Phe Thr Asp Ala Leu Gly Ile Asp Glu Tyr Gly Gly Lys
65 70 75 80

CTT CTA GGT ACC TCT AGA AGG ATA TCG AAT TCT ATA TCT TCA GGT CGC 288
Leu Leu Gly Thr Ser Arg Arg Ile Ser Asn Ser Ile Ser Ser Gly Arg
85 90 95

GAT GAT CTC TCG CCT TCG AGC AGC TTG AAC GGA TAC TCG GCG AAC GAA 336
Asp Asp Leu Ser Pro Ser Ser Ser Leu Asn Gly Tyr Ser Ala Asn Glu
100 105 110

AGC TGC GAT GCG AAG AAG AGC AAG AAG GGA CCT GCG CCA CGG GTG CAA 384
Ser Cys Asp Ala Lys Lys Ser Lys Lys Gly Pro Ala Pro Arg Val Gln
115 120 125

GAG GAG CTG TGC CTG GTT TGC GGC GAC AGG GCC TCC GGC TAC CAC TAC 432
Glu Glu Leu Cys Leu Val Cys Gly Asp Arg Ala Ser Gly Tyr His Tyr
130 135 140

AAC GCC CTC ACC TGT GAG GGC TGC AAG GGG TTC TTT CGA CGC AGC GTT 480
Asn Ala Leu Thr Cys Glu Gly Cys Lys Gly Phe Phe Arg Arg Ser Val
145 150 155 160

ACG AAG AGC GCC GTC TAC TGC TGC AAG TTC GGG CGC GCC TGC GAA ATG 528
Thr Lys Ser Ala Val Tyr Cys Cys Lys Phe Gly Arg Ala Cys Glu Met
165 170 175

GAC ATG TAC ATG AGG CGA AAG TGT CAG GAG TGC CGC CTG AAA AAG TGC 576
Asp Met Tyr Met Arg Arg Lys Cys Gln Glu Cys Arg Leu Lys Lys Cys
180 185 190

CTG GCC GTG GGT ATG CGG CCG GAA TGC GTC GTC CCG GAG AAC CAA TGT 624
Leu Ala Val Gly Met Arg Pro Glu Cys Val Val Pro Glu Asn Gln Cys
195 200 205

GCG ATG AAG CGG CGC GAA AAG AAG GCC CAG AAG GAG AAG GAC AAA ATG 672
Ala Met Lys Arg Arg Glu Lys Lys Ala Gln Lys Glu Lys Asp Lys Met
210 215 220

ACC ACT TCG CCG AGC TCT CAG CAT GGC GGC AAT GGC AGC TTG GCC TCT 720
Thr Thr Ser Pro Ser Ser Gln His Gly Gly Asn Gly Ser Leu Ala Ser
225 230 235 240

GGT GGC GGC CAA GAC TTT GTT AAG AAG GAG ATT CTT GAC CTT ATG ACA 768
Gly Gly Gly Gln Asp Phe Val Lys Lys Glu Ile Leu Asp Leu Met Thr
245 250 255

TGC GAG CCG CCC CAG CAT GCC ACT ATT CCG CTA CTA CCT GAT GAA ATA 816
Cys Glu Pro Pro Gln His Ala Thr Ile Pro Leu Leu Pro Asp Glu Ile
260 265 270

TTG GCC AAG TGT CAA GCG CGC AAT ATA CCT TCC TTA ACG TAC AAT CAG 864
Leu Ala Lys Cys Gln Ala Arg Asn Ile Pro Ser Leu Thr Tyr Asn Gln
275 280 285

TTG GCC GTT ATA TAC AAG TTA ATT TGG TAC CAG GAT GGC TAT GAG CAG 912
Leu Ala Val Ile Tyr Lys Leu Ile Trp Tyr Gln Asp Gly Tyr Glu Gln
290 295 300

CCA TCT GAA GAG GAT CTC AGG CGT ATA ATG AGT CAA CCC GAT GAG AAC 960
Pro Ser Glu Glu Asp Leu Arg Arg Ile Met Ser Gln Pro Asp Glu Asn
305 310 315 320

GAG AGC CAA ACG GAC GTC AGC TTT CGG CAT ATA ACC GAG ATA ACC ATA 1008
Glu Ser Gln Thr Asp Val Ser Phe Arg His Ile Thr Glu Ile Thr Ile
325 330 335

CTC ACG GTC CAG TTG ATT GTT GAG TTT GCT AAA GGT CTA CCA GCG TTT 1056
Leu Thr Val Gln Leu Ile Val Glu Phe Ala Lys Gly Leu Pro Ala Phe
340 345 350

ACA AAG ATA CCC CAG GAG GAC CAG ATC ACG TTA CTA AAG GCC TGC TCG 1104
Thr Lys Ile Pro Gln Glu Asp Gln Ile Thr Leu Leu Lys Ala Cys Ser
355 360 365

TCG GAG GTG ATG ATG CTG CGT ATG GCA CGA CGC TAT GAC CAC AGC TCG 1152
Ser Glu Val Met Met Leu Arg Met Ala Arg Arg Tyr Asp His Ser Ser
370 375 380

GAC TCA ATA TTC TTC GCG AAT AAT AGA TCA TAT ACG CGG GAT TCT TAC 1200
Asp Ser Ile Phe Phe Ala Asn Asn Arg Ser Tyr Thr Arg Asp Ser Tyr
385 390 395 400

AAA ATG GCC GGA ATG GCT GAT AAC ATT GAA GAC CTG CTG CAT TTC TGC 1248
Lys Met Ala Gly Met Ala Asp Asn Ile Glu Asp Leu Leu His Phe Cys
405 410 415

CGC CAA ATG TTC TCG ATG AAG GTG GAC AAC GTC GAA TAC GCG CTT CTC 1296
Arg Gln Met Phe Ser Met Lys Val Asp Asn Val Glu Tyr Ala Leu Leu
420 425 430

ACT GCC ATT GTG ATC TTC TCG GAC CGG CCG GGC CTG GAG AAG GCC CAA 1344
Thr Ala Ile Val Ile Phe Ser Asp Arg Pro Gly Leu Glu Lys Ala Gln
435 440 445

CTA GTC GAA GCG ATC CAG AGC TAC TAC ATC GAC ACG CTA CGC ATT TAT 1392
Leu Val Glu Ala Ile Gln Ser Tyr Tyr Ile Asp Thr Leu Arg Ile Tyr
450 455 460

ATA CTC AAC CGC CAC TGC GGC GAC TCA ATG AGC CTC GTC TTC TAC GCA 1440
Ile Leu Asn Arg His Cys Gly Asp Ser Met Ser Leu Val Phe Tyr Ala
465 470 475 480

AAG CTG CTC TCG ATC CTC ACC GAG CTG CGT ACG CTG GGC AAC CAG AAC 1488
Lys Leu Leu Ser Ile Leu Thr Glu Leu Arg Thr Leu Gly Asn Gln Asn
485 490 495

GCC GAG ATG TGT TTC TCA CTA AAG CTC AAA AAC CGC AAA CTG CCC AAG 1536
Ala Glu Met Cys Phe Ser Leu Lys Leu Lys Asn Arg Lys Leu Pro Lys
500 505 510

TTC CTC GAG GAG ATC TGG GAC GTT CAT GCC ATC CCG CCA TCG GTC CAG 1584
Phe Leu Glu Glu Ile Trp Asp Val His Ala Ile Pro Pro Ser Val Gln
515 520 525

TCG CAC CTT CAG ATT ACC CAG GAG GAG AAC GAG CGT CTC GAG CGG GCT 1632
Ser His Leu Gln Ile Thr Gln Glu Glu Asn Glu Arg Leu Glu Arg Ala
530 535 540

GAG CGT ATG CGG GCA TCG GTT GGG GGC GCC ATT ACC GCC GGC ATT GAT 1680
Glu Arg Met Arg Ala Ser Val Gly Gly Ala Ile Thr Ala Gly Ile Asp
545 550 555 560

TGC GAC TCT GCC TCC ACT TCG GCG GCG GCA GCC GCG GCC CAG CAT CAG 1728
Cys Asp Ser Ala Ser Thr Ser Ala Ala Ala Ala Ala Ala Gln His Gln
565 570 575

CCT CAG CCT CAG CCC CAG CCC CAA CCC TCC TCC CTG ACC CAG AAC GAT 1776
Pro Gln Pro Gln Pro Gln Pro Gln Pro Ser Ser Leu Thr Gln Asn Asp
580 585 590

TCC CAG CAC CAG ACA CAG CCG CAG CTA CAA CCT CAG CTA CCA CCT CAG 1824
Ser Gln His Gln Thr Gln Pro Gln Leu Gln Pro Gln Leu Pro Pro Gln
595 600 605

CTG CAA GGT CAA CTG CAA CCC CAG CTC CAA CCA CAG CTT CAG ACG CAA 1872
Leu Gln Gly Gln Leu Gln Pro Gln Leu Gln Pro Gln Leu Gln Thr Gln
610 615 620

CTC CAG CCA CAG ATT CAA CCA CAG CCA CAG CTC CTT CCC GTC TCC GCT 1920
Leu Gln Pro Gln Ile Gln Pro Gln Pro Gln Leu Leu Pro Val Ser Ala
625 630 635 640

CCC GTG CCC GCC TCC GTA ACC GCA CCT GGT TCC TTG TCC GCG GTC AGT 1968
Pro Val Pro Ala Ser Val Thr Ala Pro Gly Ser Leu Ser Ala Val Ser
645 650 655

ACG AGC AGC GAA TAC ATG GGC GGA AGT GCG GCC ATA GGA CCC ATC ACG 2016
Thr Ser Ser Glu Tyr Met Gly Gly Ser Ala Ala Ile Gly Pro Ile Thr
660 665 670

CCG GCA ACC ACC AGC AGT ATC ACG GCT GCC GTT ACC GCT AGC TCC ACC 2064
Pro Ala Thr Thr Ser Ser Ile Thr Ala Ala Val Thr Ala Ser Ser Thr
675 680 685

ACA TCA GCG GTA CCG ATG GGC AAC GGA GTT GGA GTC GGT GTT GGG GTG 2112
Thr Ser Ala Val Pro Met Gly Asn Gly Val Gly Val Gly Val Gly Val
690 695 700

GGC GGC AAC GTC AGC ATG TAT GCG AAC GCC CAG ACG GCG ATG GCC TTG 2160
Gly Gly Asn Val Ser Met Tyr Ala Asn Ala Gln Thr Ala Met Ala Leu
705 710 715 720

ATG GGT GTA GCC CTG CAT TCG CAC CAA GAG CAG CTT ATC GGG GGA GTG 2208
Met Gly Val Ala Leu His Ser His Gln Glu Gln Leu Ile Gly Gly Val
725 730 735

GCG GTT AAG TCG GAG CAC TCG ACG ACT GCA TAG 2241
Ala Val Lys Ser Glu His Ser Thr Thr Ala
740 745

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 746 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met Ala Pro Pro Thr Asp Val Ser Leu Gly Asp Glu Leu His Leu Asp
1 5 10 15

Gly Glu Asp Val Ala Met Ala His Ala Asp Ala Leu Asp Asp Phe Asp
20 25 30

Leu Asp Met Leu Gly Asp Gly Asp Ser Pro Gly Pro Gly Phe Thr Pro
35 40 45

His Asp Ser Ala Pro Tyr Gly Ala Leu Asp Met Ala Asp Phe Glu Phe
50 55 60

Glu Gln Met Phe Thr Asp Ala Leu Gly Ile Asp Glu Tyr Gly Gly Lys
65 70 75 80

Leu Leu Gly Thr Ser Arg Arg Ile Ser Asn Ser Ile Ser Ser Gly Arg
85 90 95

Asp Asp Leu Ser Pro Ser Ser Ser Leu Asn Gly Tyr Ser Ala Asn Glu
100 105 110

Ser Cys Asp Ala Lys Lys Ser Lys Lys Gly Pro Ala Pro Arg Val Gln
115 120 125

Glu Glu Leu Cys Leu Val Cys Gly Asp Arg Ala Ser Gly Tyr His Tyr
130 135 140

Asn Ala Leu Thr Cys Glu Gly Cys Lys Gly Phe Phe Arg Arg Ser Val
145 150 155 160

Thr Lys Ser Ala Val Tyr Cys Cys Lys Phe Gly Arg Ala Cys Glu Met

165 170 175

Asp Met Tyr Met Arg Arg Lys Cys Gln Glu Cys Arg Leu Lys Lys Cys
180 185 190

Leu Ala Val Gly Met Arg Pro Glu Cys Val Val Pro Glu Asn Gln Cys
195 200 205

Ala Met Lys Arg Arg Glu Lys Lys Ala Gln Lys Glu Lys Asp Lys Met
210 215 220

Thr Thr Ser Pro Ser Ser Gln His Gly Gly Asn Gly Ser Leu Ala Ser
225 230 235 240

Gly Gly Gly Gln Asp Phe Val Lys Lys Glu Ile Leu Asp Leu Met Thr
245 250 255

Cys Glu Pro Pro Gln His Ala Thr Ile Pro Leu Leu Pro Asp Glu Ile
260 265 270

Leu Ala Lys Cys Gln Ala Arg Asn Ile Pro Ser Leu Thr Tyr Asn Gln
275 280 285

Leu Ala Val Ile Tyr Lys Leu Ile Trp Tyr Gln Asp Gly Tyr Glu Gln
290 295 300

Pro Ser Glu Glu Asp Leu Arg Arg Ile Met Ser Gln Pro Asp Glu Asn
305 310 315 320

Glu Ser Gln Thr Asp Val Ser Phe Arg His Ile Thr Glu Ile Thr Ile
325 330 335

Leu Thr Val Gln Leu Ile Val Glu Phe Ala Lys Gly Leu Pro Ala Phe
340 345 350

Thr Lys Ile Pro Gln Glu Asp Gln Ile Thr Leu Leu Lys Ala Cys Ser
355 360 365

Ser Glu Val Met Met Leu Arg Met Ala Arg Arg Tyr Asp His Ser Ser
370 375 380

Asp Ser Ile Phe Phe Ala Asn Asn Arg Ser Tyr Thr Arg Asp Ser Tyr
385 390 395 400

Lys Met Ala Gly Met Ala Asp Asn Ile Glu Asp Leu Leu His Phe Cys
405 410 415

Arg Gln Met Phe Ser Met Lys Val Asp Asn Val Glu Tyr Ala Leu Leu
420 425 430

Thr Ala Ile Val Ile Phe Ser Asp Arg Pro Gly Leu Glu Lys Ala Gln
435 440 445

Leu Val Glu Ala Ile Gln Ser Tyr Tyr Ile Asp Thr Leu Arg Ile Tyr
450 455 460

Ile Leu Asn Arg His Cys Gly Asp Ser Met Ser Leu Val Phe Tyr Ala
465 470 475 480

Lys Leu Leu Ser Ile Leu Thr Glu Leu Arg Thr Leu Gly Asn Gln Asn
485 490 495

Ala Glu Met Cys Phe Ser Leu Lys Leu Lys Asn Arg Lys Leu Pro Lys
500 505 510

Phe Leu Glu Glu Ile Trp Asp Val His Ala Ile Pro Pro Ser Val Gln
515 520 525

Ser His Leu Gln Ile Thr Gln Glu Glu Asn Glu Arg Leu Glu Arg Ala
530 535 540

Glu Arg Met Arg Ala Ser Val Gly Gly Ala Ile Thr Ala Gly Ile Asp
545 550 555 560

Cys Asp Ser Ala Ser Thr Ser Ala Ala Ala Ala Ala Ala Gln His Gln
565 570 575

Pro Gln Pro Gln Pro Gln Pro Gln Pro Ser Ser Leu Thr Gln Asn Asp
580 585 590

Ser Gln His Gln Thr Gln Pro Gln Leu Gln Pro Gln Leu Pro Pro Gln
595 600 605

Leu Gln Gly Gln Leu Gln Pro Gln Leu Gln Pro Gln Leu Gln Thr Gln
610 615 620

Leu Gln Pro Gln Ile Gln Pro Gln Pro Gln Leu Leu Pro Val Ser Ala
625 630 635 640

Pro Val Pro Ala Ser Val Thr Ala Pro Gly Ser Leu Ser Ala Val Ser
645 650 655

Thr Ser Ser Glu Tyr Met Gly Gly Ser Ala Ala Ile Gly Pro Ile Thr
660 665 670

Pro Ala Thr Thr Ser Ser Ile Thr Ala Ala Val Thr Ala Ser Ser Thr
675 680 685

Thr Ser Ala Val Pro Met Gly Asn Gly Val Gly Val Gly Val Gly Val
690 695 700

Gly Gly Asn Val Ser Met Tyr Ala Asn Ala Gln Thr Ala Met Ala Leu
705 710 715 720

Met Gly Val Ala Leu His Ser His Gln Glu Gln Leu Ile Gly Gly Val
725 730 735

Ala Val Lys Ser Glu His Ser Thr Thr Ala
740 745

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3126 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..3126
(D) OTHER INFORMATION: /product= "GEcR"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

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ATG GAC TCC AAA GAA TCA TTA ACT CCT GGT AGA GAA GAA AAC CCC AGC      48
Met Asp Ser Lys Glu Ser Leu Thr Pro Gly Arg Glu Glu Asn Pro Ser
 1               5                  10                  15

AGT GTG CTT GCT CAG GAG AGG GGA GAT GTG ATG GAC TTC TAT AAA ACC      96
Ser Val Leu Ala Gln Glu Arg Gly Asp Val Met Asp Phe Tyr Lys Thr
            20                  25                  30

CTA AGA GGA GGA GCT ACT GTG AAG GTT TCT GCG TCT TCA CCC TCA CTG     144
Leu Arg Gly Gly Ala Thr Val Lys Val Ser Ala Ser Ser Pro Ser Leu
        35                  40                  45

GCT GTC GCT TCT CAA TCA GAC TCC AAG CAG CGA AGA CTT TTG GTT GAT     192
Ala Val Ala Ser Gln Ser Asp Ser Lys Gln Arg Arg Leu Leu Val Asp
    50                  55                  60

TTT CCA AAA GGC TCA GTA AGC AAT GCG CAG CAG CCA GAT CTG TCC AAA     240
Phe Pro Lys Gly Ser Val Ser Asn Ala Gln Gln Pro Asp Leu Ser Lys
65                  70                  75                  80

GCA GTT TCA CTC TCA ATG GGA CTG TAT ATG GGA GAG ACA GAA ACA AAA     288
Ala Val Ser Leu Ser Met Gly Leu Tyr Met Gly Glu Thr Glu Thr Lys
                85                  90                  95

GTG ATG GCA AAT GAC CTG GGA TTC CCA CAG CAG GGC CAA ATC AGC CTT     336
Val Met Gly Asn Asp Leu Gly Phe Pro Gln Gln Gly Gln Ile Ser Leu
            100                 105                 110

TCC TCG GGG GAA ACA GAC TTA AAG CTT TTG GAA GAA AGC ATT GCA AAC     384
Ser Ser Gly Glu Thr Asp Leu Lys Leu Leu Glu Glu Ser Ile Ala Asn
        115                 120                 125

CTC AAT AGG TCG ACC AGT GTT CCA GAG AAC CCC AAG AGT TCA GCA TCC     432
Leu Asn Arg Ser Thr Ser Val Pro Glu Asn Pro Lys Ser Ser Ala Ser
    130                 135                 140
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ACT GCT GTG TCT GCT GCC CCC ACA GAG AAG GAG TTT CCA AAA ACT CAC 480
Thr Ala Val Ser Ala Ala Pro Thr Glu Lys Glu Phe Pro Lys Thr His
145 150 155 160

TCT GAT GTA TCT TCA GAA CAG CAA CAT TTG AAG GGC CAG ACT GGC ACC 528
Ser Asp Val Ser Ser Glu Gln Gln His Leu Lys Gly Gln Thr Gly Thr
165 170 175

AAC GGT GGC AAT GTG AAA TTG TAT ACC ACA GAC CAA AGC ACC TTT GAC 576
Asn Gly Gly Asn Val Lys Leu Tyr Thr Thr Asp Gln Ser Thr Phe Asp
180 185 190

ATT TTG CAG GAT TTG GAG TTT TCT TCT GGG TCC CCA GGT AAA GAG ACG 624
Ile Leu Gln Asp Leu Glu Phe Ser Ser Gly Ser Pro Gly Lys Glu Thr
195 200 205

AAT GAG AGT CCT TGG AGA TCA GAC CTG TTG ATA GAT GAA AAC TGT TTG 672
Asn Glu Ser Pro Trp Arg Ser Asp Leu Leu Ile Asp Glu Asn Cys Leu
210 215 220

CTT TCT CCT CTG GCG GGA GAA GAC GAT TCA TTC CTT TTG GAA GGA AAC 720
Leu Ser Pro Leu Ala Gly Glu Asp Asp Ser Phe Leu Leu Glu Gly Asn
225 230 235 240

TCG AAT GAG GAC TGC AAG CCT CTC ATT TTA CCG GAC ACT AAA CCC AAA 768
Ser Asn Glu Asp Cys Lys Pro Leu Ile Leu Pro Asp Thr Lys Pro Lys
245 250 255

ATT AAG GAT AAT GGA GAT CTG GTT TTG TCA AGC CCC AGT AAT GTA ACA 816
Ile Lys Asp Asn Gly Asp Leu Val Leu Ser Ser Pro Ser Asn Val Thr
260 265 270

CTG CCC CAA GTG AAA ACA GAA AAA GAA GAT TTC ATC GAA CTC TGC ACC 864
Leu Pro Gln Val Lys Thr Glu Lys Glu Asp Phe Ile Glu Leu Cys Thr
275 280 285

CCT GGG GTA ATT AAG CAA GAG AAA CTG GGC ACA GTT TAC TGT CAG GCA 912
Pro Gly Val Ile Lys Gln Glu Lys Leu Gly Thr Val Tyr Cys Gln Ala
290 295 300

AGC TTT CCT GGA GCA AAT ATA ATT GGT AAT AAA ATG TCT GCC ATT TCT 960
Ser Phe Pro Gly Ala Asn Ile Ile Gly Asn Lys Met Ser Ala Ile Ser
305 310 315 320

GTT CAT GGT GTG AGT ACC TCT GGA GGA CAG ATG TAC CAC TAT GAC ATG 1008
Val His Gly Val Ser Thr Ser Gly Gly Gln Met Tyr His Tyr Asp Met
325 330 335

AAT ACA GCA TCC CTT TCT CAA CAG CAG GAT CAG AAG CCT ATT TTT AAT 1056
Asn Thr Ala Ser Leu Ser Gln Gln Gln Asp Gln Lys Pro Ile Phe Asn
340 345 350

GTC ATT CCA CCA ATT CCC GTT GGT TCC GAA AAT TGG AAT AGG TGC CAA 1104
Val Ile Pro Pro Ile Pro Val Gly Ser Glu Asn Trp Asn Arg Cys Gln
355 360 365

GGA TCT GGA GAT GAC AAC TTG ACT TCT CTG GGG ACT CTG AAC TTC CCT 1152
Gly Ser Gly Asp Asp Asn Leu Thr Ser Leu Gly Thr Leu Asn Phe Pro
370 375 380

GGT CGA ACA GTT TTT TCT AAT GGC TAT TCA AGC CCC AGC ATG AGA CCA 1200
Gly Arg Thr Val Phe Ser Asn Gly Tyr Ser Ser Pro Ser Met Arg Pro
385 390 395 400

GAT GTA AGC TCT CCT CCA TCC AGC TCC TCA ACA GCA ACA ACA GGA CCA 1248
Asp Val Ser Ser Pro Pro Ser Ser Ser Ser Thr Ala Thr Thr Gly Pro
405 410 415

CCT CCC AGC GGC CGC GTG CAA GAG GAG CTG TGC CTG GTT TGC GGC GAC 1296
Pro Pro Ser Gly Arg Val Gln Glu Glu Leu Cys Leu Val Cys Gly Asp
420 425 430

AGG GCC TCC GGC TAC CAC TAC AAC GCC CTC ACC TGT GGA TCC TGC AAG 1344
Arg Ala Ser Gly Tyr His Tyr Asn Ala Leu Thr Cys Gly Ser Cys Lys
435 440 445

GTG TTC TTT CGA CGC AGC GTT ACG AAG AGC GCC GTC TAC TGC TGC AAG 1392
Val Phe Phe Arg Arg Ser Val Thr Lys Ser Ala Val Tyr Cys Cys Lys
450 455 460

TTC GGG CGC GCC TGC GAA ATG GAC ATG TAC ATG AGG CGA AAG TGT CAG 1440
Phe Gly Arg Ala Cys Glu Met Asp Met Tyr Met Arg Arg Lys Cys Gln
465 470 475 480

GAG TGC CGC CTG AAA AAG TGC CTG GCC GTG GGT ATG CGG CCG GAA TGC 1488
Glu Cys Arg Leu Lys Lys Cys Leu Ala Val Gly Met Arg Pro Glu Cys
485 490 495

GTC GTC CCG GAG AAC CAA TGT GCG ATG AAG CGG CGC GAA AAG AAG GCC 1536
Val Val Pro Glu Asn Gln Cys Ala Met Lys Arg Arg Glu Lys Lys Ala
500 505 510

CAG AAG GAG AAG GAC AAA ATG ACC ACT TCG CCG AGC TCT CAG CAT GGC 1584
Gln Lys Glu Lys Asp Lys Met Thr Thr Ser Pro Ser Ser Gln His Gly
515 520 525

GGC AAT GGC AGC TTG GCC TCT GGT GGC GGC CAA GAC TTT GTT AAG AAG 1632
Gly Asn Gly Ser Leu Ala Ser Gly Gly Gly Gln Asp Phe Val Lys Lys
530 535 540

GAG ATT CTT GAC CTT ATG ACA TGC GAG CCG CCC CAG CAT GCC ACT ATT 1680
Glu Ile Leu Asp Leu Met Thr Cys Glu Pro Pro Gln His Ala Thr Ile
545 550 555 560

CCG CTA CTA CCT GAT GAA ATA TTG GCC AAG TGT CAA GCG CGC AAT ATA 1728
Pro Leu Leu Pro Asp Glu Ile Leu Ala Lys Cys Gln Ala Arg Asn Ile
565 570 575

CCT TCC TTA ACG TAC AAT CAG TTG GCC GTT ATA TAC AAG TTA ATT TGG 1776
Pro Ser Leu Thr Tyr Asn Gln Leu Ala Val Ile Tyr Lys Leu Ile Trp
580 585 590

TAC CAG GAT GGC TAT GAG CAG CCA TCT GAA GAG GAT CTC AGG CGT ATA 1824
Tyr Gln Asp Gly Tyr Glu Gln Pro Ser Glu Glu Asp Leu Arg Arg Ile
595 600 605

ATG AGT CAA CCC GAT GAG AAC GAG AGC CAA ACG GAC GTC AGC TTT CGG 1872
Met Ser Gln Pro Asp Glu Asn Glu Ser Gln Thr Asp Val Ser Phe Arg
610 615 620

CAT ATA ACC GAG ATA ACC ATA CTC ACG GTC CAG TTG ATT GTT GAG TTT 1920
His Ile Thr Glu Ile Thr Ile Leu Thr Val Gln Leu Ile Val Glu Phe
625 630 635 640

GCT AAA GGT CTA CCA GCG TTT ACA AAG ATA CCC CAG GAG GAC CAG ATC 1968
Ala Lys Gly Leu Pro Ala Phe Thr Lys Ile Pro Gln Glu Asp Gln Ile
645 650 655

ACG TTA CTA AAG GCC TGC TCG TCG GAG GTG ATG ATG CTG CGT ATG GCA 2016
Thr Leu Leu Lys Ala Cys Ser Ser Glu Val Met Met Leu Arg Met Ala
660 665 670

CGA CGC TAT GAC CAC AGC TCG GAC TCA ATA TTC TTC GCG AAT AAT AGA 2064
Arg Arg Tyr Asp His Ser Ser Asp Ser Ile Phe Phe Ala Asn Asn Arg
675 680 685

TCA TAT ACG CGG GAT TCT TAC AAA ATG GCC GGA ATG GCT GAT AAC ATT 2112
Ser Tyr Thr Arg Asp Ser Tyr Lys Met Ala Gly Met Ala Asp Asn Ile
690 695 700

GAA GAC CTG CTG CAT TTC TGC CGC CAA ATG TTC TCG ATG AAG GTG GAC 2160
Glu Asp Leu Leu His Phe Cys Arg Gln Met Phe Ser Met Lys Val Asp
705 710 715 720

AAC GTC GAA TAC GCG CTT CTC ACT GCC ATT GTG ATC TTC TCG GAC CGG 2208
Asn Val Glu Tyr Ala Leu Leu Thr Ala Ile Val Ile Phe Ser Asp Arg
725 730 735

CCG GGC CTG GAG AAG GCC CAA CTA GTC GAA GCG ATC CAG AGC TAC TAC 2256
Pro Gly Leu Glu Lys Ala Gln Leu Val Glu Ala Ile Gln Ser Tyr Tyr
740 745 750

ATC GAC ACG CTA CGC ATT TAT ATA CTC AAC CGC CAC TGC GGC GAC TCA 2304
Ile Asp Thr Leu Arg Ile Tyr Ile Leu Asn Arg His Cys Gly Asp Ser
755 760 765

ATG AGC CTC GTC TTC TAC GCA AAG CTG CTC TCG ATC CTC ACC GAG CTG 2352
Met Ser Leu Val Phe Tyr Ala Lys Leu Leu Ser Ile Leu Thr Glu Leu
770 775 780

CGT ACG CTG GGC AAC CAG AAC GCC GAG ATG TGT TTC TCA CTA AAG CTC 2400
Arg Thr Leu Gly Asn Gln Asn Ala Glu Met Cys Phe Ser Leu Lys Leu
785 790 795 800

AAA AAC CGC AAA CTG CCC AAG TTC CTC GAG GAG ATC TGG GAC GTT CAT 2448
Lys Asn Arg Lys Leu Pro Lys Phe Leu Glu Glu Ile Trp Asp Val His
805 810 815

GCC ATC CCG CCA TCG GTC CAG TCG CAC CTT CAG ATT ACC CAG GAG GAG 2496
Ala Ile Pro Pro Ser Val Gln Ser His Leu Gln Ile Thr Gln Glu Glu
820 825 830

AAC GAG CGT CTC GAG CGG GCT GAG CGT ATG CGG GCA TCG GTT GGG GGC 2544
Asn Glu Arg Leu Glu Arg Ala Glu Arg Met Arg Ala Ser Val Gly Gly
835 840 845

GCC ATT ACC GCC GGC ATT GAT TGC GAC TCT GCC TCC ACT TCG GCG GCG 2592
Ala Ile Thr Ala Gly Ile Asp Cys Asp Ser Ala Ser Thr Ser Ala Ala
850 855 860

GCA GCC GCG GCC CAG CAT CAG CCT CAG CCT CAG CCC CAG CCC CAA CCC 2640
Ala Ala Ala Ala Gln His Gln Pro Gln Pro Gln Pro Gln Pro Gln Pro
865 870 875 880

TCC TCC CTG ACC CAG AAC GAT TCC CAG CAC CAG ACA CAG CCG CAG CTA 2688
Ser Ser Leu Thr Gln Asn Asp Ser Gln His Gln Thr Gln Pro Gln Leu
885 890 895

CAA CCT CAG CTA CCA CCT CAG CTG CAA GGT CAA CTG CAA CCC CAG CTC 2736
Gln Pro Gln Leu Pro Pro Gln Leu Gln Gly Gln Leu Gln Pro Gln Leu
900 905 910

CAA CCA CAG CTT CAG ACG CAA CTC CAG CCA CAG ATT CAA CCA CAG CCA 2784
Gln Pro Gln Leu Gln Thr Gln Leu Gln Pro Gln Ile Gln Pro Gln Pro
915 920 925

CAG CTC CTT CCC GTC TCC GCT CCC GTG CCC GCC TCC GTA ACC GCA CCT 2832
Gln Leu Leu Pro Val Ser Ala Pro Val Pro Ala Ser Val Thr Ala Pro
930 935 940

GGT TCC TTG TCC GCG GTC AGT ACG AGC AGC GAA TAC ATG GGC GGA AGT 2880
Gly Ser Leu Ser Ala Val Ser Thr Ser Ser Glu Tyr Met Gly Gly Ser
945 950 955 960

GCG GCC ATA GGA CCC ATC ACG CCG GCA ACC ACC AGC AGT ATC ACG GCT 2928
Ala Ala Ile Gly Pro Ile Thr Pro Ala Thr Thr Ser Ser Ile Thr Ala
965 970 975

GCC GTT ACC GCT AGC TCC ACC ACA TCA GCG GTA CCG ATG GGC AAC GGA 2976
Ala Val Thr Ala Ser Ser Thr Thr Ser Ala Val Pro Met Gly Asn Gly
980 985 990

GTT GGA GTC GGT GTT GGG GTG GGC GGC AAC GTC AGC ATG TAT GCG AAC 3024
Val Gly Val Gly Val Gly Val Gly Gly Asn Val Ser Met Tyr Ala Asn
995 1000 1005

GCC CAG ACG GCG ATG GCC TTG ATG GGT GTA GCC CTG CAT TCG CAC CAA 3072
Ala Gln Thr Ala Met Ala Leu Met Gly Val Ala Leu His Ser His Gln
1010 1015 1020

GAG CAG CTT ATC GGG GGA GTG GCG GTT AAG TCG GAG CAC TCG ACG ACT 3120
Glu Gln Leu Ile Gly Gly Val Ala Val Lys Ser Glu His Ser Thr Thr
1025 1030 1035 1040

GCA TAG 3126
Ala

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1041 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Met Asp Ser Lys Glu Ser Leu Thr Pro Gly Arg Glu Glu Asn Pro Ser
1 5 10 15

Ser Val Leu Ala Gln Glu Arg Gly Asp Val Met Asp Phe Tyr Lys Thr
20 25 30

Leu Arg Gly Gly Ala Thr Val Lys Val Ser Ala Ser Ser Pro Ser Leu
35 40 45

Ala Val Ala Ser Glu Ser Asp Ser Lys Gln Arg Arg Leu Leu Val Asp
50 55 60

Phe Pro Lys Gly Ser Val Ser Asn Ala Gln Gln Pro Asp Leu Ser Lys
65 70 75 80

Ala Val Ser Leu Ser Met Gly Leu Tyr Met Gly Glu Thr Glu Thr Lys
85 90 95

Val Met Gly Asn Asp Leu Gly Phe Pro Gln Gln Gly Gln Ile Ser Leu
100 105 110

Ser Ser Gly Glu Thr Asp Leu Lys Leu Leu Glu Glu Ser Ile Ala Asn
115 120 125

Leu Asn Arg Ser Thr Ser Val Pro Glu Asn Pro Lys Ser Ser Ala Ser
130 135 140

Thr Ala Val Ser Ala Ala Pro Thr Glu Lys Glu Phe Pro Lys Thr His
145 150 155 160

Ser Asp Val Ser Ser Glu Gln Gln His Leu Lys Gly Gln Thr Gly Thr
165 170 175

Asn Gly Gly Asn Val Lys Leu Tyr Thr Thr Asp Gln Ser Thr Phe Asp
180 185 190

Ile Leu Gln Asp Leu Glu Phe Ser Ser Gly Ser Pro Gly Lys Glu Thr
195 200 205

Asn Glu Ser Pro Trp Arg Ser Asp Leu Leu Ile Asp Glu Asn Cys Leu
210 215 220

Leu Ser Pro Leu Ala Gly Glu Asp Asp Ser Phe Leu Leu Glu Gly Asn
225 230 235 240

Ser Asn Glu Asp Cys Lys Pro Leu Ile Leu Pro Asp Thr Lys Pro Lys
245 250 255

Ile Lys Asp Asn Gly Asp Leu Val Leu Ser Ser Pro Ser Asn Val Thr
260 265 270

Leu Pro Gln Val Lys Thr Glu Lys Glu Asp Phe Ile Glu Leu Cys Thr
275 280 285

Pro Gly Val Ile Lys Gln Glu Lys Leu Gly Thr Val Tyr Cys Gln Ala
290 295 300

Ser Phe Pro Gly Ala Asn Ile Ile Gly Asn Lys Met Ser Ala Ile Ser
305 310 315 320

Val His Gly Val Ser Thr Ser Gly Gly Gln Met Tyr His Tyr Asp Met
325 330 335

Asn Thr Ala Ser Leu Ser Gln Gln Gln Asp Gln Lys Pro Ile Phe Asn
340 345 350

Val Ile Pro Pro Ile Pro Val Gly Ser Glu Asn Trp Asn Arg Cys Gln
355 360 365

Gly Ser Gly Asp Asp Asn Leu Thr Ser Leu Gly Thr Leu Asn Phe Pro
370 375 380

Gly Arg Thr Val Phe Ser Asn Gly Tyr Ser Ser Pro Ser Met Arg Pro
385 390 395 400

Asp Val Ser Ser Pro Pro Ser Ser Ser Ser Thr Ala Thr Thr Gly Pro
405 410 415

Pro Pro Ser Gly Arg Val Gln Glu Glu Leu Cys Leu Val Cys Gly Asp
420 425 430

Arg Ala Ser Gly Tyr His Tyr Asn Ala Leu Thr Cys Gly Ser Cys Lys
435 440 445

Val Phe Phe Arg Arg Ser Val Thr Lys Ser Ala Val Tyr Cys Cys Lys
450 455 460

Phe Gly Arg Ala Cys Glu Met Asp Met Tyr Met Arg Arg Lys Cys Gln
465 470 475 480

Glu Cys Arg Leu Lys Lys Cys Leu Ala Val Gly Met Arg Pro Glu Cys
485 490 495

Val Val Pro Glu Asn Gln Cys Ala Met Lys Arg Arg Glu Lys Lys Ala
500 505 510

Gln Lys Glu Lys Asp Lys Met Thr Thr Ser Pro Ser Ser Gln His Gly
515 520 525

Gly Asn Gly Ser Leu Ala Ser Gly Gly Gly Gln Asp Phe Val Lys Lys
530 535 540

Glu Ile Leu Asp Leu Met Thr Cys Glu Pro Pro Gln His Ala Thr Ile
545 550 555 560

Pro Leu Leu Pro Asp Glu Ile Leu Ala Lys Cys Gln Ala Arg Asn Ile
565 570 575

Pro Ser Leu Thr Tyr Asn Gln Leu Ala Val Ile Tyr Lys Leu Ile Trp
580 585 590

Tyr Gln Asp Gly Tyr Glu Gln Pro Ser Glu Glu Asp Leu Arg Arg Ile
595 600 605

Met Ser Gln Pro Asp Glu Asn Glu Ser Gln Thr Asp Val Ser Phe Arg
610 615 620

His Ile Thr Glu Ile Thr Ile Leu Thr Val Gln Leu Ile Val Glu Phe
625 630 635 640

Ala Lys Gly Leu Pro Ala Phe Thr Lys Ile Pro Gln Glu Asp Gln Ile
645 650 655

Thr Leu Leu Lys Ala Cys Ser Ser Glu Val Met Met Leu Arg Met Ala
660 665 670

Arg Arg Tyr Asp His Ser Ser Asp Ser Ile Phe Phe Ala Asn Asn Arg
675 680 685

Ser Tyr Thr Arg Asp Ser Tyr Lys Met Ala Gly Met Ala Asp Asn Ile
690 695 700

Glu Asp Leu Leu His Phe Cys Arg Gln Met Phe Ser Met Lys Val Asp
705 710 715 720

Asn Val Glu Tyr Ala Leu Leu Thr Ala Ile Val Ile Phe Ser Asp Arg
725 730 735

Pro Gly Leu Glu Lys Ala Gln Leu Val Glu Ala Ile Gln Ser Tyr Tyr
740 745 750

Ile Asp Thr Leu Arg Ile Tyr Ile Leu Asn Arg His Cys Gly Asp Ser
755 760 765

Met Ser Leu Val Phe Tyr Ala Lys Leu Leu Ser Ile Leu Thr Glu Leu
770 775 780

Arg Thr Leu Gly Asn Gln Asn Ala Glu Met Cys Phe Ser Leu Lys Leu
785 790 795 800

Lys Asn Arg Lys Leu Pro Lys Phe Leu Glu Glu Ile Trp Asp Val His
805 810 815

Ala Ile Pro Pro Ser Val Gln Ser His Leu Gln Ile Thr Gln Glu Glu
820 825 830

Asn Glu Arg Leu Glu Arg Ala Glu Arg Met Arg Ala Ser Val Gly Gly
835 840 845

Ala Ile Thr Ala Gly Ile Asp Cys Asp Ser Ala Ser Thr Ser Ala Ala
850 855 860

Ala Ala Ala Ala Gln His Gln Pro Gln Pro Gln Pro Gln Pro Gln Pro
865 870 875 880

Ser Ser Leu Thr Gln Asn Asp Ser Gln His Gln Thr Gln Pro Gln Leu
885 890 895

Gln Pro Gln Leu Pro Pro Gln Leu Gln Gly Gln Leu Gln Pro Gln Leu
900 905 910

Gln Pro Gln Leu Gln Thr Gln Leu Gln Pro Gln Ile Gln Pro Gln Pro
915 920 925

Gln Leu Leu Pro Val Ser Ala Pro Val Pro Ala Ser Val Thr Ala Pro
930 935 940

Gly Ser Leu Ser Ala Val Ser Thr Ser Ser Glu Tyr Met Gly Gly Ser
945 950 955 960

Ala Ala Ile Gly Pro Ile Thr Pro Ala Thr Thr Ser Ser Ile Thr Ala
965 970 975

Ala Val Thr Ala Ser Ser Thr Thr Ser Ala Val Pro Met Gly Asn Gly
980 985 990

Val Gly Val Gly Val Gly Val Gly Gly Asn Val Ser Met Tyr Ala Asn
995 1000 1005

Ala Gln Thr Ala Met Ala Leu Met Gly Val Ala Leu His Ser His Gln
1010 1015 1020

Glu Gln Leu Ile Gly Gly Val Ala Val Lys Ser Glu His Ser Thr Thr
1025 1030 1035 1040

Ala

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 7
(D) OTHER INFORMATION: /product= "Modified Ecdysone Response Element"
/note= "N at position 7 is 0 up to 5 nucleotides, with 1 nucleotide being especially preferred."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

RGBNNMNTGN NCY 13

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 7
(D) OTHER INFORMATION: /product= "Modified Ecdysone Response Element"
/note= "N at position 7 can be 0 up to 5 nucleotides, with 1 nucleotide being preferred."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

RGNNCANKNN VCY 13

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AGTGCANTGT TCT 13

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 7
(D) OTHER INFORMATION: /product= "Ecdysone Response Element"
/note= "N at position 7 can be 0 up to 5 nucleotides, with 3 nucleotides being preferred."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

RGBNNMNRGB NNM 13

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TACAACGCCC TCACCTGTGG ATCCTGCAAG GTGTTTCTTT CGACGCAGC 49

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GTACTCCCGG GGCGGGGCTA TGCGGGGCGG GGCTAATCGC TAGGGGCGGG GCA 53

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GTACTGCCCC GCCCCTAGCG ATTAGCCCCG CCCCGCATAG CCCCGCCCCG GGA 53

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AGCTCGATGG ACAAGTGCAT TGTTCTTTGC TGAA 34

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AGCTTTCAGC AAGAGAACAA TGCACTTGTC CATCG 35

That which is claimed is:

1. A method for modulating the expression of an exogenous gene in a mammalian subject containing:

(i) a DNA construct comprising said exogenous gene under the control of an ecdysone response element; and

(ii) a modified ecdysone receptor which, in the presence of a ligand therefor, and optionally in the further presence of a receptor capable of acting as a silent partner therefor, binds to said ecdysone response element;

said method comprising administering to said subject an effective amount of a ligand for said modified ecdysone receptor; wherein said ligand is not normally present in the cells of said subject; and wherein said ligand is not toxic to said subject.

2. A method according to claim 1 wherein said modified ecdysone receptor comprises:

a ligand binding domain capable of binding an ecdysteroid;

a DNA-binding domain obtained from a DNA-binding protein; and

an activation domain of a transcription factor,

wherein at least one of said DNA-binding domain or said activation domain is not obtained from a native ecdysone receptor,

with the proviso that when said activation domain is derived from a glucocorticoid receptor, said DNA-binding domain is not derived from a glucocorticoid receptor or an *E. coli* LexA protein.

3. A method according to claim 2 wherein said modified ecdysone receptor is further characterized as having substantially no constitutive activity in mammalian cells.

4. A method according to claim 2 wherein the DNA-binding domain of said modified ecdysone receptor is derived from a member of the steroid/thyroid hormone superfamily of receptors.

5. A method according to claim 2 wherein said activation domain is obtained from a member of the steroid/thyroid hormone superfamily of receptors.

6. A method according to claim 2 wherein said activation domain is selected from a glucocorticoid receptor activation domain, a VP16 activation domain or a GAL4 activation domain.

7. A method according to claim 6 wherein said modified ecdysone receptor is selected from VpEcR, VgEcR or GEcR.

8. A method according to claim 7 wherein said modified ecdysone receptor is VgEcR having the amino acid sequence set forth in SEQ ID NO:5.

9. A method according to claim 1 wherein said modified ecdysone receptor is present primarily in the form of a homodimer.

10. A method according to claim 9 wherein said ecdysone response element is the native ecdysone response element.

11. A method according to claim 1 wherein said receptor capable of acting as a silent partner is RXR.

12. A method according to claim 11 wherein said RXR is exogenous to said mammalian subject.

13. A method according to claim 1 wherein said ecdysone response element is a modified response element which comprises, in any order, a first half-site and a second half-site separated by a spacer of 0-5 nucleotides;

wherein said first half-site has the sequence:

-RGBNNM-,

wherein

each R is independently selected from A or G;

each B is independently selected from G, C, or T;

each N is independently selected from A, T, C, or G; and

each M is independently selected from A or C;

with the proviso that at least 4 nucleotides of each -RGBNNM- group of nucleotides are identical with the nucleotides at comparable positions of the sequence -AGGTCA-; and

said second half-site is obtained from a glucocorticoid receptor subfamily response element.

14. A method according to claim 13 wherein said response element has substantially no binding affinity for farnesoid X receptor (FXR).

15. A method according to claim 1 wherein said ligand is a naturally occurring ecdysone, an ecdysone-analog or an ecdysone mimic.

16. A method according to claim 15 wherein said naturally occurring ecdysone is α-ecdysone or β-ecdysone.

17. A method according to claim 15 wherein said ecdysone analog is ponasterone A, ponasterone B, ponasterone C, 26-iodoponasterone A, muristerone A, inokosterone or 26-mesylinokosterone.

18. A method according to claim 15 wherein said ecdysone mimic is 3,5-di-tert-butyl-4-hydroxy-N-isobutyl-benzamide, 8-O-acetylharpagide, a 1,2-diacyl hydrazine, an N'-substituted-N,N'-disubstituted hydrazine, a dibenzoylalkyl cyanohydrazine, an N-substituted-N-alkyl-N,N-diaroyl hydrazine, an N-substituted-N-acyl-N-alkyl, carbonyl hydrazine or an N-aroyl-N'-alkyl-N'-aroyl hydrazine.

19. A method according to claim 1 wherein said exogenous gene is a wild type gene and/or therapeutic gene.

20. A method according to claim 19 wherein said wild type gene is selected from genes which encode products:

the substantial absence of which leads to the occurrence of a non-normal state in said subject; or

a substantial excess of which leads to the occurrence of a non-normal state in said subject.

21. A method according to claim 19 wherein said therapeutic gene is selected from those which encode products:

which are toxic to the cells in which they are expressed; or

which impart a beneficial property to said subject.

22. A method of inducing the expression of an exogenous gene in a mammalian subject containing:

(i) a DNA construct comprising an exogenous gene under the control of an ecdysone response element,

(ii) DNA encoding a modified ecdysone receptor under the control of an inducible promoter; wherein said modified ecdysone receptor, in the presence of a ligand therefor, and optionally in the further presence of a receptor capable of acting as a silent partner therefor, binds to said ecdysone response element, and

(iii) a ligand for said modified ecdysone receptor;

said method comprising subjecting said subject to conditions suitable to induce expression of said modified ecdysone receptor.

23. A method of inducing expression of an exogenous gene in a mammalian subject containing a DNA construct containing said exogenous gene under the control of an ecdysone response element, said method comprising introducing into said subject:

a modified ecdysone receptor; and

a ligand for said modified ecdysone receptor,

wherein said receptor, in combination with a ligand therefor, and optionally in the further presence of a receptor capable of acting as a silent partner therefor, binds to said ecdysone response element, activating transcription therefrom.

24. A method for the expression of a recombinant product detrimental to a host organism, said method comprising:

transforming suitable host cells with:

(i) a DNA construct encoding said recombinant product under the control of an ecdysone response element, and

(ii) DNA encoding a modified ecdysone receptor;

growing said host cells in suitable media; and

inducing expression of said recombinant product by introducing into said host cells ligand(s) for said modified ecdysone receptor, and optionally a receptor capable of acting as a silent partner for said modified ecdysone receptor.

25. A pharmaceutically acceptable formulation comprising at least one ecdysteroid and a pharmaceutically acceptable carrier.

26. A formulation according to claim 25 wherein said pharmaceutically acceptable carrier renders said formulation suitable for oral, topical, nasal, transdermal, intravenous, subcutaneous, intramuscular, intracutaneous, intraperitoneal or intravascular administration.

27. A formulation according to claim 25 wherein said ecdysteroid is a naturally occurring ecdysone, an ecdysone-analog or an ecdysone mimic.

28. A formulation according to claim 27 wherein said naturally occurring ecdysone is α-ecdysone or β-ecdysone.

29. A formulation according to claim 27 wherein said ecdysone analog is ponasterone A, ponasterone B, ponasterone C, 26-iodoponasterone A, muristerone A, inokosterone or 26-mesylinokosterone.

30. A formulation according to claim 27 wherein said ecdysone mimic is 3,5-di-tert-butyl-4-hydroxy-N-isobutyl-benzamide, 8-O-acetylharpagide, a 1,2-diacyl hydrazine, an N'-substituted-N,N'-disubstituted hydrazine, a dibenzoylalkyl cyanohydrazine, an N-substituted-N-alkyl-N,N-diaroyl hydrazine, an N-substituted-N-acyl-N-alkyl, carbonyl hydrazine or an N-aroyl-N'-alkyl-N'-aroyl hydrazine.

31. A pharmaceutically acceptable formulation consisting essentially of at least one ecdysteroid and a pharmaceutically acceptable carrier.

32. A formulation according to claim 31 wherein said ecdysteroid is a naturally occurring ecdysone, an ecdysone-analog or an ecdysone mimic.

33. A kit comprising at least one ecdysteroid and a pharmaceutically acceptable carrier therefor.

34. A formulation according to claim 33 wherein said ecdysteroid is a naturally occurring ecdysone, an ecdysone-analog or an ecdysone mimic.

ABSTRACT OF THE DISCLOSURE

In accordance with the present invention, there are provided various methods for modulating the expression of an exogenous gene in a mammalian subject employing modified ecdysone receptors. Also provided are modified ecdysone receptors, as well as homomeric and heterodimeric receptors containing same, nucleic acids encoding invention modified ecdysone receptors, modified ecdysone response elements, gene transfer vectors, recombinant cells, and transgenic animals containing nucleic acids encoding invention modified ecdysone receptor.

DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION

As the below-named inventor(s), we hereby declare that:

Our residence, post office address and citizenship are as stated below next to our name.

We believe we are the original, first and joint inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled METHODS FOR MODULATING EXPRESSION OF EXOGENOUS GENES IN MAMMALIAN SYSTEMS, AND PRODUCTS RELATED THERETO the specification of which

__X__ is attached hereto.

____ was filed on _____ (Attorney Docket No. _____) as Application Serial No. _____ and was amended on (or amended through) _____. (if applicable)

I authorize and request insertion of the serial number of the application when officially known:

United States Serial No.: _____

We hereby state that we have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to above.

We acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Sec. 1.56(a).

We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further

that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

We hereby appoint the following attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

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Date: _________________

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Figure 1A

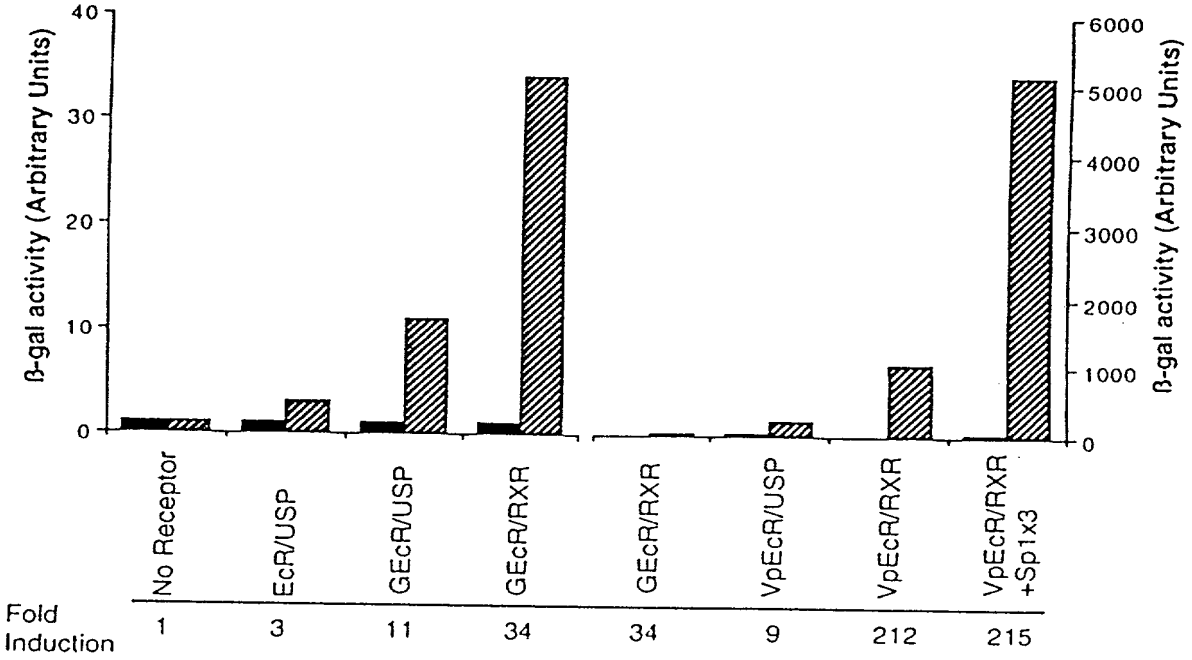
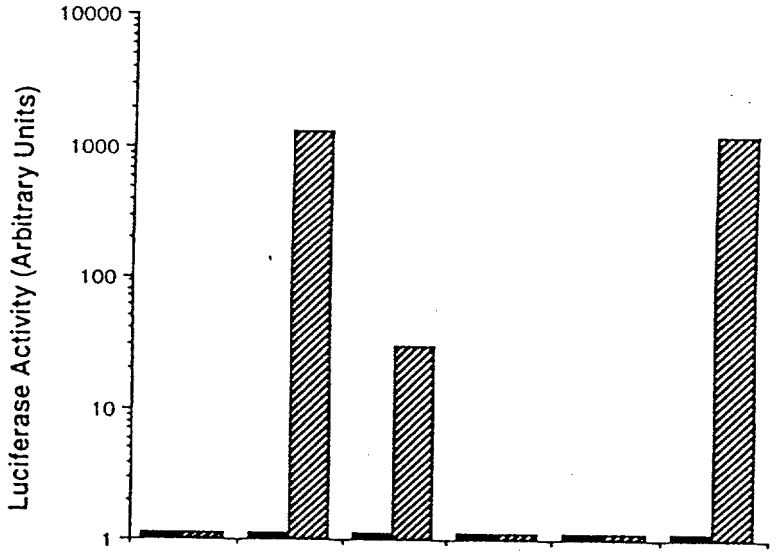


Figure 1B



	-	VpEcR RXR	FXR RXR	VpEcR RXR	FXR RXR	VgEcR RXR
Reporter	EcRE	EcRE	EcRE	E/GRE	E/GRE	E/GRE
Fold Induction	1	1293	29	1	1	1201

Figure 1C

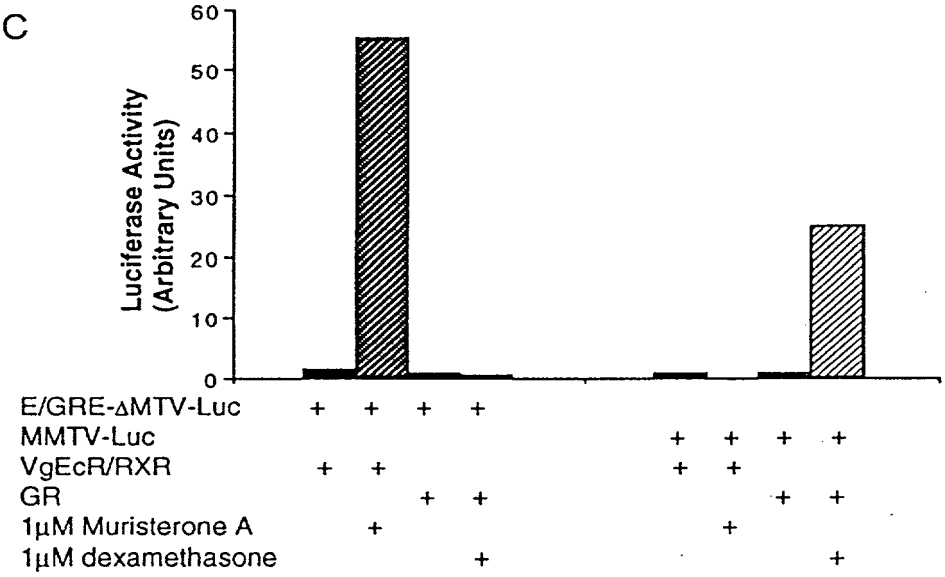
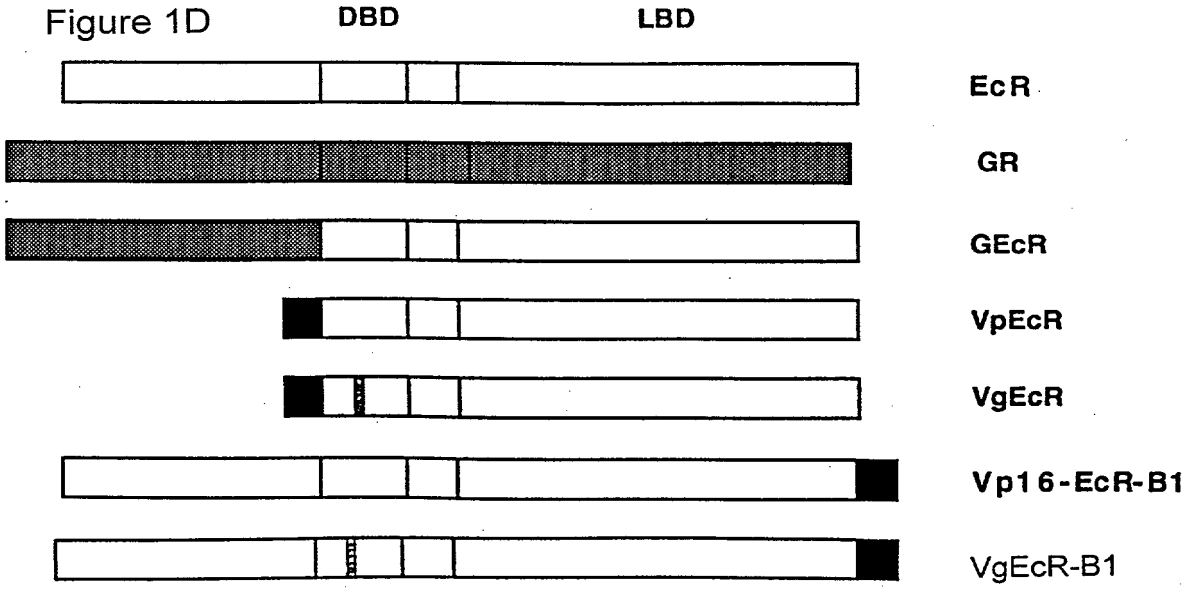


Figure 1D



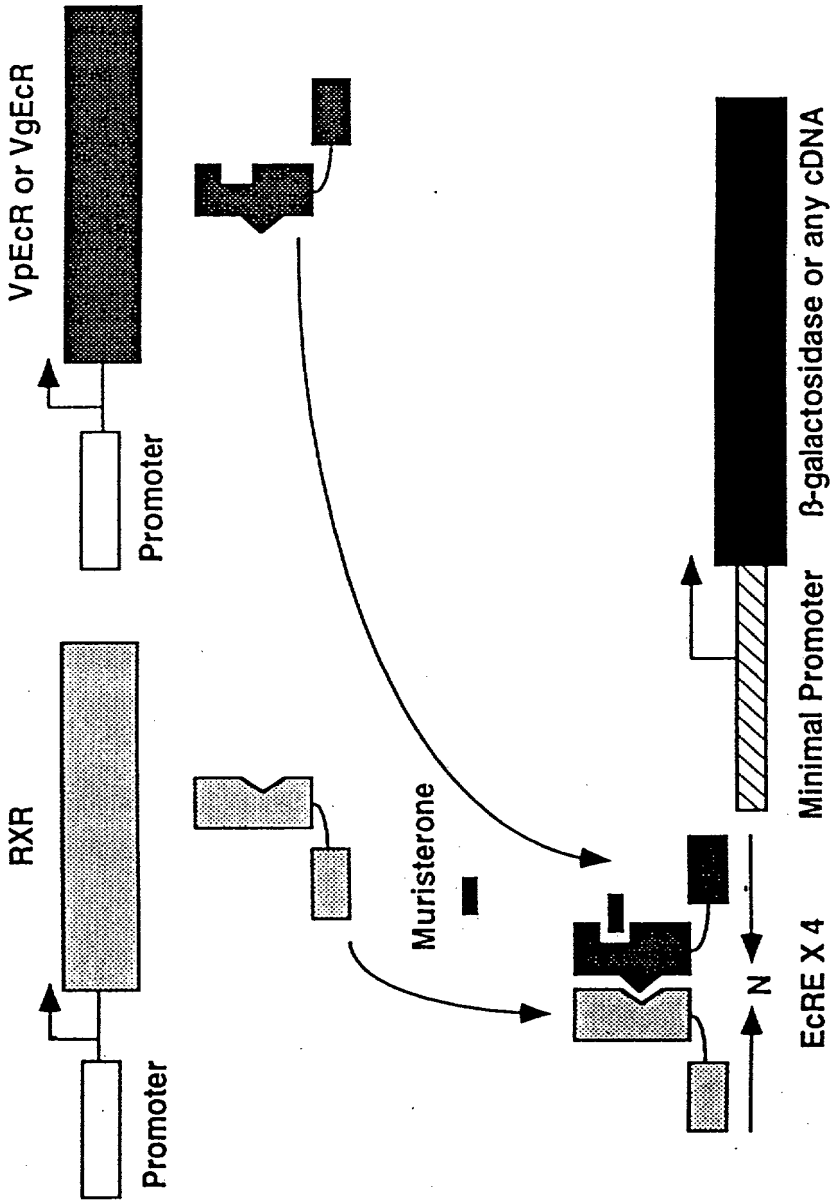

VpEcR or VgEcR
Promoter
RXR
Promoter
Muristerone
ß-galactosidase or any cDNA
Minimal Promoter
EcRE X 4
N

Figure 2

Figure 3A

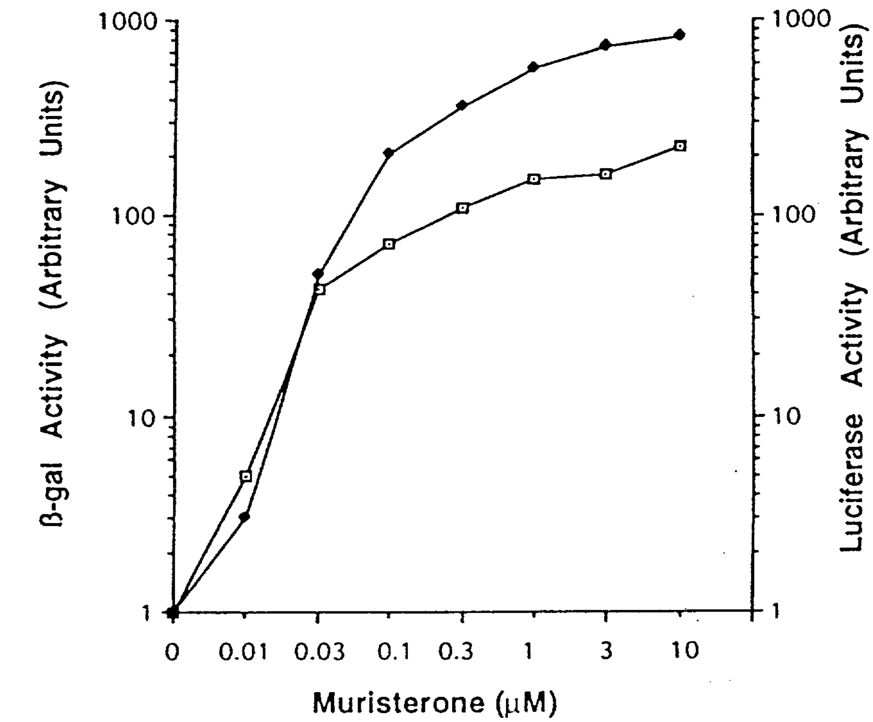


Figure 3B

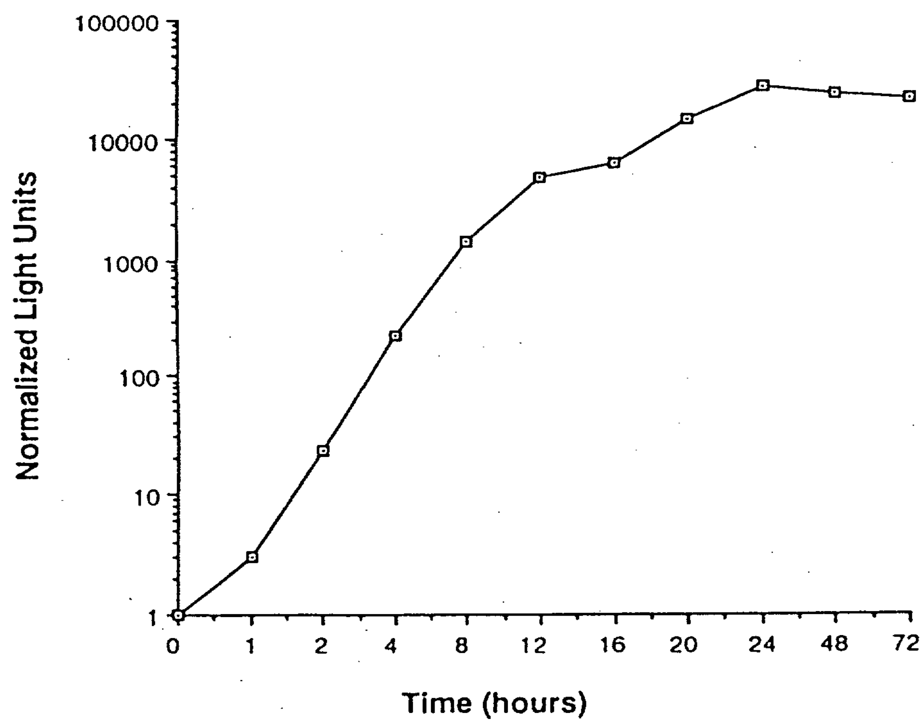
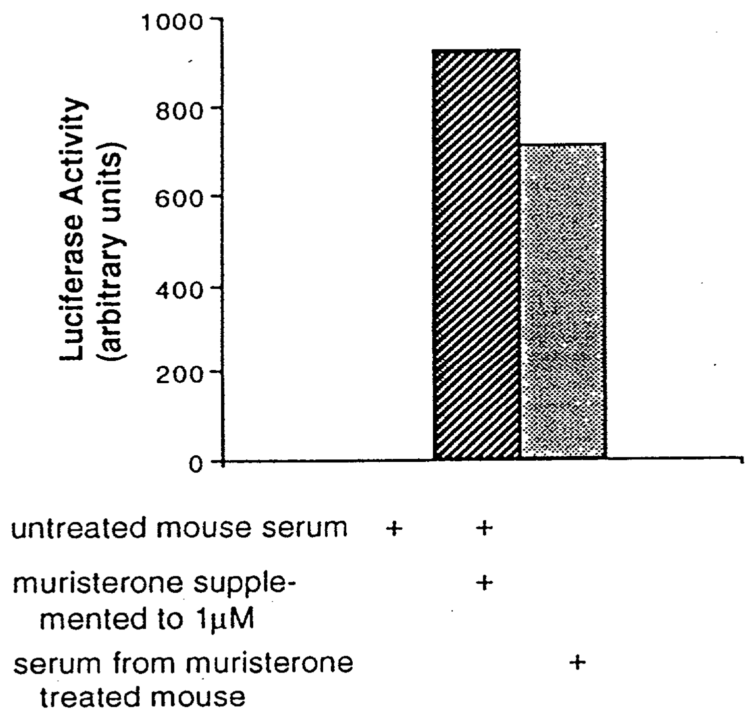


Figure 4


Luciferase Activity
(arbitrary units)
1000
800
600
400
200
0
untreated mouse serum + +
muristerone supple-
mented to 1μM +
serum from muristerone
treated mouse +

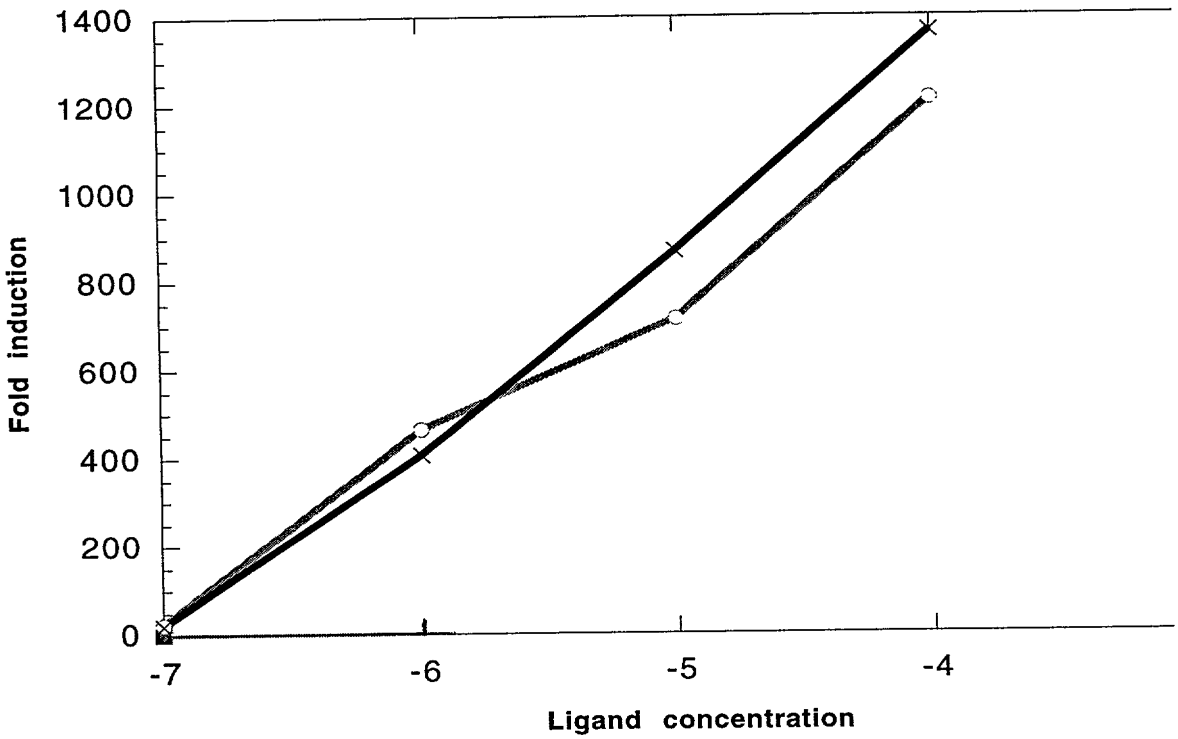

1400
1200
1000
800
600
400
200
0
Fold induction
-7
-6
-5
-4
Ligand concentration

Figure 5